

REMARKS

A petition for a one month extension of time has today been filed as a separate paper and a copy is attached hereto.

Any fees required under 37 CFR 1.17(p) should be charged to deposit account number 12-2174.

Regarding the amendments to claim 1, note that in the original specification at page 2, line 24, the term “second subloop” is defined as “UPL”. That this “UPL” is composed of 11 consecutive amino acids is evidenced by: Biochemical and Biophysical Research Communications 285, 1309-1316 (2001), a copy of which is attached hereto, along with a form PTO-A820. Note, especially, the teaching at page 1309, left column, lines 4-9:

“two pentapeptides(S₁₆₉-Q₁₇₀-K₁₇₁-E₁₇₂-G₁₇₃ of CCR5 and E₁₇₉-A₁₈₀-D₁₈₁-D₁₈₂-R₁₈₃ of CXCR4) which are components of the undeca-peptidyl arch (UPA: from R₁₆₈ to C₁₇₈ in CCR5, from N₁₇₆ to C₁₈₆ in CXCR4) of extracellular loop 2 (ECL2) in chemokine receptors (CCR5 and CXCR4).”

The acronym “UPL” in the present specification is an abbreviation for “undeca-peptidyl loop” and has the same meaning as the “UPA” in the above-cited publication and thus means a sequence consisting of 11 consecutive amino acids.

Responsive to paragraph 3 of the office action submitted herewith are listings of

the references submitted with the Information Disclosure Statement filed October 30, 2001. Applicants' file indicates that these lists were in fact filed with the submission on October 30, 2001.

Responsive to paragraphs 4 and 6 of the office action, a Substitute Specification and Abstract is submitted herewith. The Substitute Specification contains no new matter. In order that the examiner can satisfy himself in this regard, also submitted herewith is a marked-up copy of the original specification and abstract, from which the Substitute Specification and Abstract was typed. The examiner will note that a separate paragraph has been inserted at the top of page 1 of the specification as suggested by the examiner in paragraph 4 of the office action. The examiner will also note that sequence identifiers have been inserted throughout the specification as required in paragraph 6 of the office action.

Responsive to paragraph 5 of the office action, submitted herewith is a "Sequence Listing" as page 16 of the Substitute Specification and in computer readable form on a floppy disk. On information and belief, the content of the sequence listing information recorded in computer readable form on the floppy disk submitted herewith is identical to that of the written paper form submitted here as a new page 16 of the substitute specification. Further, the sequence listing here contains no new matter, having been fully disclosed in the original specification.

The rejection of claims 1 and 4-6 for indefiniteness, as set forth in paragraph 8 of the office action is at least partially moot in view of the present amendments which substitute in part the language suggested by the examiner. At page 2, lines 19-22 applicants' identify the second receptors of T-cells and macrophages as CXCR4 and CCR5, respectively. Given the knowledge in the art with regard to the amino acid structure of the second extracellular domains of the CXCR4 and CCR5 receptors (again, see the first-listed reference submitted here), one skilled in the art would be able to determine whether or not a given amino acid sequence (1) comprises at least 5 consecutive amino acids and (2) is contained within the 11 consecutive amino acids of the second extracellular loop of CXCR4 or CCR5. In other words, one skilled in the art would be able to determine whether or not a given amino acid sequence within a cyclic peptide does or does not satisfy claim 1. Nothing more is required by the second paragraph of 35 USC 112. The second paragraph of 35 USC 112 has been construed as requiring no more than sufficient preciseness that one skilled in the art can determine what does and what does not fall within the scope of the claim. *In re Conley*, 180 USPQ 454 at 456 (CCPA 1974). No amount of "breadth" can substantiate a rejection for indefiniteness under the second paragraph of 35 USC 112. *In re Goffe*, 188 USPQ 131 (CCPA 1975) and *Ex parte Scherberich*, 201 USPQ 397 (PTO Bd. App. 1977).

Accordingly, the examiner's questions posed in paragraph 8 of the office action are irrelevant to any issue of definiteness. Specifically, how the peptides are cyclized is not relevant. If the peptides are not cyclized, they do not fall within the scope of

claim 1. If they are cyclized they fall within the scope of claim 1, provided they meet the other requirements of claim 1. There is no indefiniteness, a peptide is either cyclized or is not cyclized. Likewise, the examiner's questions as to which amino acid residues are involved, his question as to which portions of the peptide can be modified to accommodate substituent groups (claims 4 and 5) and his question regarding the protection of side groups are all irrelevant to a consideration of any issue of definiteness. For example, the recitation of claim 5 is satisfied wherein one of the recited substituent groups is bonded through any one or more of the active groups recited by claim 4. Again, it is either is or is not so bonded as required by claims 4 and 5. There is no in-between and there is no indefiniteness. The question "Are any of the side groups protected by chemical substituents and unavailable for further modification?" is of no relevance to a determination of what does and does not fall within the scope of claim 1.

The examiner's questions as set forth in paragraph 9 of the office action seem to relate to the process by which the claimed peptides are produced and it is not understood how the question relate to an issue of definiteness in the sense of the capability of one skilled in the art to determine what does and what does not fall within the scope of claims 2 and 7. Claim 2 simply requires that a cyclic peptide as otherwise defined by claim 1 includes one of the two specifically recited 5 amino acid sequences.

Regarding paragraph 10 of the office action, a "peptide", by definition, is "a compound consisting of two or more amino acids linked covalently through peptide

bonds,” quoting from page 259 Coombs, Dictionary of Biotechnology (2nd Edition).

That definition would apply to both linear and cyclic peptides. There is no reason to believe that one skilled in the art would interpret the structure of claim 3 as having any type of bonding other than peptide bonding.

The Rejection Under the First Paragraph of 35 USC 112 - Paragraph 12 of the Office Action

1. The Examiner's remarks that the "disclosure fails to identify suitable peptides that can reasonably be expected to provide a therapeutic or protective effect against HIV infection" are inapplicable to composition Claims 1 and 2. To the contrary, the Examiner's attention is respectfully drawn to Disclosure page 6, lines 2 through 12 where in vitro tests and an inhibitory effect against HIV is specifically stated.

As noted in *In re Cortright*, 49 USPQ2d 1464 (Fed.Cir. 1999), the PTO cannot make this type of rejection, however, unless it has reason to doubt the objective truth of the statements contained in the written description. *See Brana*, 51 F.3d at 1566, 34 USPQ2d at 1441:

[T]he PTO has the initial burden of challenging a presumptively correct assertion of utility in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility." (citations omitted).

2. Disclosure of Immunogens:

Claimed compositions are drawn to peptide sites that result in a biological response. Claimed vaccines are clearly defined. The Examiner's attention is again respectfully drawn to the specification at page 6, lines 2 through 12 where *in vitro* tests and an inhibitory effect against HIV is specifically stated, as well as to Fig. 1 where a peptide structure is defined.

3. Working Embodiments

The Examiner has questioned the amount or quality of the *in vivo* data. *In vitro* data is presented (Disclosure page 6, lines 2 through 12, page 15, 11 through 19). It is respectfully suggested that such data fully and unarguably satisfies the utility and enablement requirements for patentability. *In re Gottlieb et al*, 140 USPQ 665 (CCPA 1964). Applicants respectfully suggest that, while useful, a standard of "art-recognized" as to animal models is not an absolute requirement in establishing utility. Certainly, in the absence of evidence or apparent reason why the invention does not perform as claimed, the allegation of utility in the Specification must be accepted as correct. *In re Bundy*, 209 USPQ 48 (CCPA 1981). The presumption is, thus, in favor of utility, in the absence of contrary data.

4. State of the Art HIV

The Examiner's statement that "there is no effective vaccine for the prevention or

treatment of HIV-1 or HIV-2 infection" is not a statement of the requirements of patentability. Nor is this a statement of fact.

The Specification at page 6, line 11 teaches an "inhibitory effect against HIV infection." Attached here to is an abstract as published in Entrez Pubmed "On the analysis of viral load endpoints in HIV vaccine trials." Hudgens *et al.*, Stat Med. 2003 Vol. 30;22(14):2281-98. This abstract states that "differences in viral loads between these groups would suggest a vaccine effect on disease progression." This is consistent with Applicant's disclosure of "inhibitory effect against HIV infection."

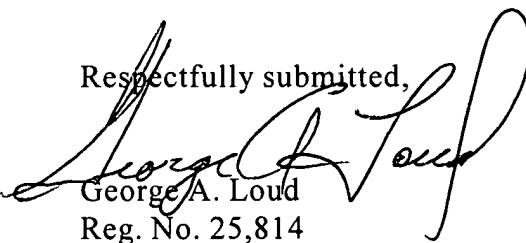
In the absence of evidence or apparent reason why the invention does not perform as claimed, the allegation of utility in the Specification must be accepted as correct. *In re Bundy*, 209 USPQ 48 (CCPA 1981). The presumption is, thus, in favor of utility, in the absence of contrary data.

The examiner is also asked to consider that in the original specification, page 12. under (4), applicants disclose that monoclonal antibody-producing hybridoma was prepared by immunizing Balb/c mice using the cyclic dodecapeptide-MAP as immunogens and under (5), it is disclosed that in vitro anti-HIV activity was measured. In the document submitted here from Biochemical and Biophysical Research Communications 285, 1309-1316 (2001) published after filing of the instant application the inventors (Applicants) disclosed similar experiments.

Further, after the filing of the instant application. the present inventors (Applicants) carried out the same anti-HIV activity measurements as disclosed in the instant specification in cynomolgus monkeys using the cyclic peptides of the present invention. Thus, in The Journal of Biological Chemistry, Vol. 278, No. 34, issue of August 22, pp. 32335-32343, they reported that cDDX4-specific antibodies having anti-HIV activity are induced by immunization of cynomolgus monkeys. A copy of that publication is also submitted herewith.

In conclusion, it is respectfully requested that the examiner reconsider the rejections of record with a view toward allowance of the claims as amended.

Respectfully submitted,



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Dated: November 3, 2003

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INFORMATION DISCLOSURE CITATION

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U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

			Biochemical and Biophysical Research Communications 285, 1309-1316 (2001)
			The Journal of Biological Chemistry, Vol. 278, No. 34, Issue of August 22, pp. 32335-32343, 2003

EXAMINER

DATE CONSIDERED

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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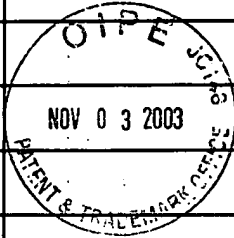
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		Stat Med. 2003 Jul30; 22(14):2281-98, On the analysis of viral load endpoints in HIV vaccine trials
		Japan Pharmacology Academy Kyushu Branch Mass Meeting Lecture Summary Collection, pp 43

EXAMINER

DATE CONSIDERED

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On the analysis of viral load endpoints in HIV vaccine trials.

Hudgens MG, Hoering A, Self SG.

Statistical Center For HIV/AIDS Research and Prevention, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N, MW-500, P.O. Box 19024, Seattle, WA 98109, USA. mhudgens@sharp.org

First generation HIV vaccines are not likely to provide complete protection from HIV-1 infection. Therefore, it is important to assess a vaccine's effect on disease progression and infectiousness of infected vaccinees in an efficacy trial; however, direct assessment of such vaccine effects is not feasible within current trial designs. Viral load in HIV-infected individuals correlates with infectiousness and disease progression in a natural history setting, and thus is a reasonable candidate for a surrogate outcome in vaccine efficacy trials. We consider comparisons of viral load of infected vaccinees to that of infected trial participants in the control group. Dramatic differences in viral loads between these groups would suggest a vaccine effect on disease progression. However, modest differences, even if statistically significant, could be consistent with an imperfect vaccine effect on susceptibility to infection and not an effect on disease progression, that is, a selection effect of the vaccine. Thus, the usual statistical tests for no difference between groups do not test the biologically and clinically relevant hypothesis. We propose a model for the possible selective effects of a vaccine and develop several test statistics for assessing a direct effect of the vaccine on viral load given this selection model. Finite sample properties of these tests are evaluated using computer simulations. Copyright 2003 John Wiley & Sons, Ltd.

PMID: 12854093 [PubMed - in process]

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=12854093&dopt=Abstract

Evidence as a HIV-1 Self-Defense Vaccine of Cyclic Chimeric Dodecapeptide Warped from Undecapeptidyl Arch of Extracellular Loop 2 in Both CCR5 and CXCR4¹

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Novel conformation-specific antibodies were raised against a cyclic chimeric dodecapeptidyl multiple antigen peptide (cCD-MAP) constructed with a spacer-armed Gly-Asp dipeptide and two pentapeptides (S₁₆₉-Q₁₇₀-K₁₇₁-E₁₇₂-G₁₇₃ of CCR5 and E₁₇₉-A₁₈₀-D₁₈₁-D₁₈₂-R₁₈₃ of CXCR4) which are components of the undecapeptidyl arch (UPA: from R₁₆₈ to C₁₇₈ in CCR5, from N₁₇₆ to C₁₈₆ in CXCR4) of extracellular loop 2 (ECL2) in chemokine receptors (CCR5 and CXCR4). Of the antibodies raised, one monoclonal antibody, CPMab-I (IgM κ), reacted with cCD-MAP, but not with the linear chimeric dodecapeptide-MAP. The antibody reacted with the cells separately expressing CCR5 or CXCR4, but not with those not expressing the coreceptors. Moreover, the antibody markedly suppressed infection by X4, R5, or R5X4 virus in a dose-dependent manner in a new phenotypic assay for drug susceptibility of HIV-1 using CCR5-expressing Hela/CD4⁺ cell clone 1-10 (MAGIC-5). Moreover, CPMab-I interfered with LAV-1_{BRU} infection (m.o.i. = 0.01) of Molt4#8 cells cocultured with CPMab-I-producing hybridoma in the transwell, and significantly interfered with neither chemotaxis nor calcium influx induced with stromal cell-derived factor 1 α (SDF-1 α). Thus, the antibody raised against the cCD-MAP provides powerful protection or defense against HIV-1 infection. We therefore propose the cCD-MAP or its derivative immunogen as a novel can-

didate for an HIV-1 coreceptor-based self-defense vaccine. © 2001 Academic Press

Key Words: chemokine receptors; CCR5; CXCR4; HIV-1 coreceptor-based self-defense vaccine.

The breakthrough in HIV-1 vaccines has been disturbed by the ineffectivity of recombinant-HIV-protein-based vaccines because of the poor immunogenicity of HIV envelope glycoproteins, the resistance of HIV-1 to neutralizing antibodies, the extensive variation in the HIV-1 genome, and the ability of the virus to become integrated in the host genome of immune cells (1, 2). There is, therefore, an urgent need for an effective vaccine against HIV-1.

We have therefore pursued a different approach by developing a new HIV-1 defense vaccine based on self-proteins, CCR5, and CXCR4, because the generation of autoantibodies against HLA class I, CD4, and CCR5 has been reported in the sera of individuals who remain HIV-seronegative and are apparently uninfected despite multiple exposures to HIV-1; these antibodies are known to block HIV-1 infection *in vitro* (3, 4, 5). Recent studies have reported that (i) the chemokine receptors, CCR5 and CXCR4, are considered to be the major coreceptors in HIV-1 infection, (ii) the evolution of HIV-1 towards using CXCR4 is potentially enhanced under conditions in which CCR5 is blocked by a CCR5 antagonist (6), (iii) selective blockade of the CXCR4 receptor prevents the switch from the less pathogenic R5 HIV-1 to the more pathogenic X4 HIV-1 (7), and (iv) CCR5 is considered as a redundant molecule in adults because CCR5-defective individuals were described to have normal inflammatory and immune reactions (8). Therefore, chemokine receptors, CCR5 and CXCR4, may become important targets for receptor antagonists involving specific mAbs against native receptors.

Abbreviations used: HIV-1, human immunodeficiency virus type 1; cCD-MAP, cyclic chimeric dodecapeptidyl multiple antigen peptide; UPA, undecapeptidyl arch; ECL2, extracellular loop 2; SDF-1, stromal cell-derived factor 1 α ; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

¹ This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Ever since chemokine receptors, CCR5 and CXCR4, were successively identified as HIV coreceptors in 1996, their functions were determined by recombinant DNA technologies (9–16). The function of ECL-2 of CCR5 and CXCR4 was determined to be critical for virus entry and signaling (10, 14, 15, 17); in particular, the undcapeptidyl arch (UPA: from N₁₇₆ to C₁₈₆ in CXCR4, from R₁₆₈ to C₁₇₈ in CCR5) of ECL-2 is shown to be very critical for HIV-1 infection. Therefore, we attempted to create the cCD-MAP to develop specific mAbs that concomitantly block both CCR5 and CXCR4 for effective therapy. The cCD-MAP is constructed with the cyclic bound form of two pentapeptides (S₁₆₉-Q₁₇₀-K₁₇₁-E₁₇₂-G₁₇₃ and E₁₇₉-A₁₈₀-D₁₈₁-D₁₈₂-R₁₈₃ in UPA of CCR5 and CXCR4) as a potent HIV-1-coreceptor-based chimeric antigen.

In this study, we demonstrate that cCD-MAP can induce novel conformation-specific antibodies, and that an induced antibody concurrently reacts with cells expressing the native human CCR5 or CXCR4. We also propose the possibility of using HIV-coreceptor-based self-defense vaccines in place of virus-based vaccines to overcome the unprecedented scientific obstacles in the development of HIV-1 vaccines.

MATERIALS AND METHODS

Preparation of cyclic chimeric dodecapeptide-MAP and -Multi-Pin Block. The all-side-chain-group-protected dodecapeptide (HOOC-G₁R₂D₃D₄A₅E₆G₇E₈K₉Q₁₀S₁₁D₁₂-NH₂) was synthesized from Gly₁ of the spacer-armed dipeptide to the pentapeptide, RDDAE, of CXCR4, followed by GEQKS of CCR5, yielding Asp₁₂ of the spacer-armed dipeptide using an automatic peptide synthesizer. The product obtained was cyclized by bond formation between the α -carboxyl group of Gly₁ and the α -amino group of Asp₁₂ in the protected dodecapeptide. The *o*-benzyl group of the β -carboxyl group of Asp₁₂ in the protected dodecapeptide was selectively removed by reduction of paladium (0.5%) on carbon. The identity of the purified cyclic chimeric dodecapeptide (cCD) was confirmed by determining their molecular mass using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS, Bruker Franzen Analytik GmbH, Bremen, Germany).

The deprotected β -carboxyl group of Asp₁₂ in the spacer-armed dipeptidyl portion of the protected cCD was separately conjugated with MAP-resin (Applied Biosystems Japan Ltd., Japan) and Multi-Pin-Block according to the manufacturer's instructions (Chiron Technologies, Australia). Then, it was deprotected or cleaved from the resin by a conventional method. The cCD-MAP and -Multi-Pin-Block were respectively used in the immunization of mice and in an enzyme-linked immunosorbent assay (ELISA) for screening monoclonal antibodies produced in hybridomas.

MALDI-TOF MS analyses for a synthetic cyclic chimeric dodecapeptide. The identity of the purified cCD was reconfirmed by determining its molecular mass using a MALDI-TOF MS, which was operated at a wavelength of 337 nm. The ion-accelerating voltage was 19.0 kV. The reflectron voltage was set at 20.0 kV. The spectra were calculated by external calibration using [M + H]⁺ ions produced from angiotensin II (*m/z* 1047.20) and substance P (*m/z* 1348.74). The matrix was a saturated solution of α -cyano-4-hydroxycinnamic acid in a solution of 1:2 acetonitrile/water containing 0.1% trifluoroacetic acid.

Preparation of anti-cCD-MAP mAbs. Female Balb/c mice were immunized intraperitoneally with cCD-MAP in Freund's adjuvant at

1-week intervals and administered an intravenous boost of cCD-MAP 3 days prior to splenectomy. Hybridomas were generated by a standard method, by which splenocytes were fused with the P3U1 cells, and selected in hypoxanthine-, aminopterin-, and thymidine-supplemented medium. In the screening, supernatants were tested for reactivity to cCD-Multi-Pin-Block. Hybridomas that produced the most potent supernatants were then cloned by limiting dilution.

ELISA. First, 200 μ l of a precoat buffer (0.01 M phosphate-buffered saline (PBS) containing 2% w/v bovine serum albumin, 0.1% v/v Tween 20, and 0.1% w/v sodium azide; pH 7.2) was dispensed into each well of microtiter plates. The cCD-Multi-Pin-Block was placed in the wells, and incubated for 60 min at room temperature. After precoating and washing with PBS, 100 μ l/well of an antibody solution, and 50 μ l/well of effector solutions were dispensed into the microtiter plates. Then, the cCD-Multi-Pin-Block was placed in the wells, and incubated for 60 min at room temperature. After four washings with PBS, 150 μ l of 1:5000-diluted peroxidase-conjugated goat anti-mouse IgM (Jackson Immuno Research) was added to the wells and incubated for 60 min at room temperature. The cCD-Multi-Pin-Block was washed four times with PBS. A substrate buffer containing 3,3',5,5'-tetramethylbenzidine and H₂O₂ was then added to the wells, and the absorbance of the solution was determined at 450/630 nm.

Flow cytometry. The following Abs were used: CPMab-I, an isotype-matched control antibody (Sigma Chemical Co., St. Louis, MO), and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM.

NP2/CD4, NP2/CD4/CXCR4, and NP2/CD4/CCR5 (18) were incubated with CPMab-I as the primary antibody at 4°C, washed with a washing buffer (PBS containing 2% fetal calf serum (FCS), and 0.02% NaN₃) and then resuspended in the washing buffer containing FITC-conjugated anti-mouse IgM. After 30 min of incubation at 4°C, the cells were washed three times, and then analyzed using an EPICS XL flow cytometer (Beckman Coulter).

Antiviral activities. The neutralizing activity of the antibodies was determined using MAGIC-5 cells, which were engineered to express CCR5 in HeLa-CD4-LTR/ β -gal cells by transfection with an expression vector for CCR5 (19). The cells are susceptible to infection by X4, R5, and R5X4 viruses. The MAGIC-5 cells were plated at 1×10^4 cells per well (48-well plate) and incubated overnight in a culture medium (200 μ l); the medium was then changed with a CPMab-I-containing medium ($1 \times$: 400 ng/ml, twofold serial dilution). The cells were then separately incubated with various HIV-1 strains (200 μ l of X4, R5, or R5X4 virus suspension) containing a HIV-1 p24 antigen (5.6 to 280 ng) in the presence of DEAE dextran (20 μ g/ml) for 2 h, washed twice with the culture medium, and cocultured in the CPMab-I-containing medium ($1 \times$: 400 ng/ml, twofold serial dilution) for an additional 48 h. The cells were fixed, and HIV-1-infected cells, stained in blue, were counted by conventional methods. Control experiments were carried out under identical conditions, except for omission of the antibody.

Coculture and infection. The cocultivation was carried out in the transwell to show whether CPMab-I inhibited HIV-1 infection via CXCR4 without affecting the CXCR4 signaling responsible for cellular functions. In the lower chamber, Molt4#8 cells (3×10^5) were incubated in the presence or absence of Hyb-I (1×10^5), which was placed in the upper chamber of the 3- μ m-pore-size transwell (Corning, NY). In control experiments, Molt4#8 cells were also incubated in the presence of control hybridoma Hyb-p2, which produces an anti-HIV-1p24^{gag} monoclonal antibody (IgMk). After coculture for 24 h, Molt4#8 cells were infected with HIV-1 (multiplicity of infection (m.o.i.) = 0.01) or inoculated into the RPMI1640 medium, and then incubated with Hyb-I or Hyb-p2 at 37°C for an additional 72 h. Molt4#8 cells were harvested, and the detection of HIV-1 proviral DNA was carried out by PCR.

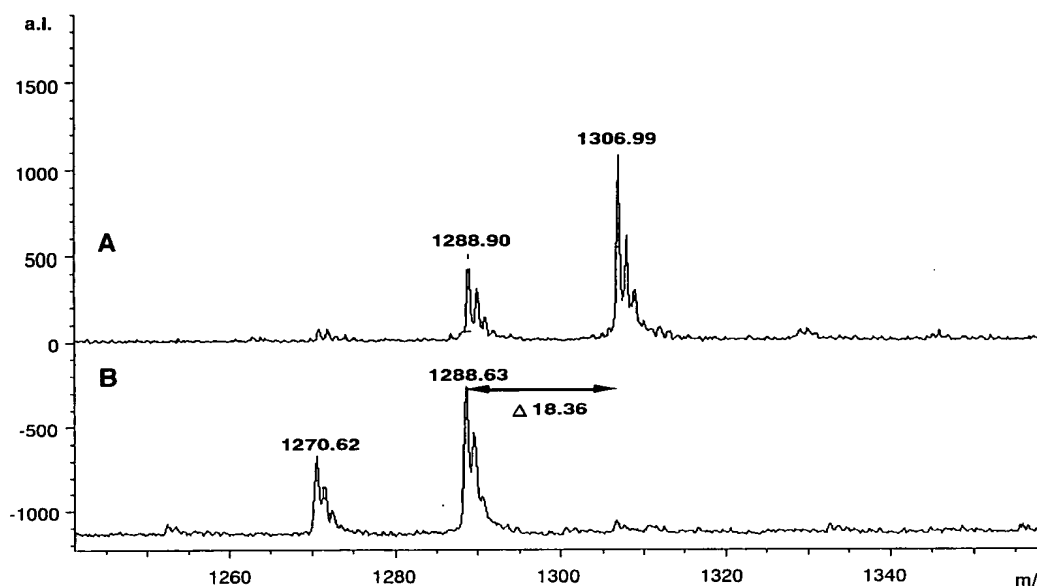


FIG. 1. MALDI TOF MS spectra for a synthetic cCD. The spectra exhibited two major peaks at m/z 1288.63 $[M + H - H_2O]^+$ and 1306.99 $[M + H]^+$; the former peak is that of the ion derived from cCD, DSQKEGEADDRG and the latter peak is that of the ion derived from the linear CD, DSQKEGEADDRG. The two peaks at m/z 1270.62 and 1288.90 are those of the dehydrated forms of the cyclic and linear peptide, respectively, produced by laser irradiation (337 nm) of MALDI-TOF MS.

Detection of HIV-1 proviral DNA by PCR. After coculture for 96 h, Molt4#8 cells were harvested. The total nucleic acid obtained after the purification procedure (20) was used for the PCR amplification according to the manufacturer's instruction (Perkin-Elmer Corp., Norwalk, CT). The following primer pairs were used: gag-specific primers (SK38 and SK39) and β -actin-specific primers (XAH-17 and XAH-20). The PCR was carried out in a thermal cycler using the following amplification cycle: 1 cycle, 94°C for 5 min; 35 cycles, 94°C for 1 min; 60°C for 1 min; 72°C for 1 min; 1 cycle, 72°C for 5 min. The PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Chemotaxis assay. The chemotaxis assay was performed using the protocol of Gosling *et al.* with modifications (21). Briefly, Molt4#8 cells cocultured with Hyb-I for 96 h were loaded into the upper chamber of the 5- μ m-pore-size transwell (Corning, NY). The chemotaxis assay was conducted in the presence of 10 nM SDF-1 α (Pepro Tech EC Ltd., London, England) placed in the lower chamber. The transwells were incubated for 3 h at 37°C. The cells that migrated from the upper to the lower chamber were quantified by trypan blue dye exclusion.

Calcium influx assay using a flow cytometer. The intracellular calcium concentration was monitored to investigate whether CPMab-I interferes with the SDF-1 α -induced calcium influx in Molt4#8 cells according to the manufacturer's instruction (EPICS XL; Beckman Coulter). Molt4#8 cells were exposed to 1 μ g of 12G5 or CPMab-I for 24 h. The cells were incubated with 100 μ M Fluo-3, AM (Molecular Probes, Inc.) for 30 min at 37°C. The cells were washed twice and incubated for another 30 min. The fluorescence (λ_{ex} 506 nm, λ_{em} 525 nm) of Fluo-3, AM-loaded cells in direct response to SDF-1 α (5 nM) was monitored using a flow cytometer according to the manufacturer's instructions (Beckman Coulter).

RESULTS

Preparation of cCD-MAP and -Multi-Pin Block

The cCD (cyclized at the head and tail of the linear CD) is constructed with the cyclic bound form of two

pentapeptides (both S₁₆₉-Q₁₇₀-K₁₇₁-E₁₇₂-G₁₇₃ and E₁₇₉-A₁₈₀-D₁₈₁-D₁₈₂-R₁₈₃ derived from UPA of ECL-2 of CCR5 and CXCR4) and the spacer-armed Gly-Asp dipeptide (22). After removing the resin, both the linear CD, DSQKEGEADDRG and the cCD, DSQKEGEADDRG were purified by HPLC, and their molecular masses determined by MALDI-TOF MS using α -cyano-4-hydroxy-cinnamic acid as a matrix. The spectra of the purified linear CD and cCD exhibited major peaks at m/z 1306.99 (the upper spectrum of Fig. 1) and 1288.63 (the lower spectrum of Fig. 1), respectively. Aliquots of the protected cCD with the free β -carboxyl group of Asp₁ were used for the construction of cCD-MAP (for immunization as an antigen or competitor) and cCD-Multi-Pin-Block (antibody screening assay for ELISA).

Immunochemical Specificity of the Conformational Specific Monoclonal Antibody

Of the antibodies produced, one monoclonal antibody, CPMab-I (IgM κ), was selected as a specific antibody against cCD-Multi-Pin-Block. As shown in Fig. 2, the immunochemical reaction was inhibited with 1 μ mol of cCD-MAP, but not with both the linear CD-MAP and MAP treated with glycidol to protect amino groups. Moreover, the binding activity of the antibody to cells expressing CCR5 or CXCR4 was determined using a flow cytometer. The antibody evidently reacted with either the CCR5- or CXCR4-expressing NP-2/CD4 cells (Figs. 3B and 3C), but with neither CCR5- nor CXCR4-nonexpressing cells (Fig. 3A).

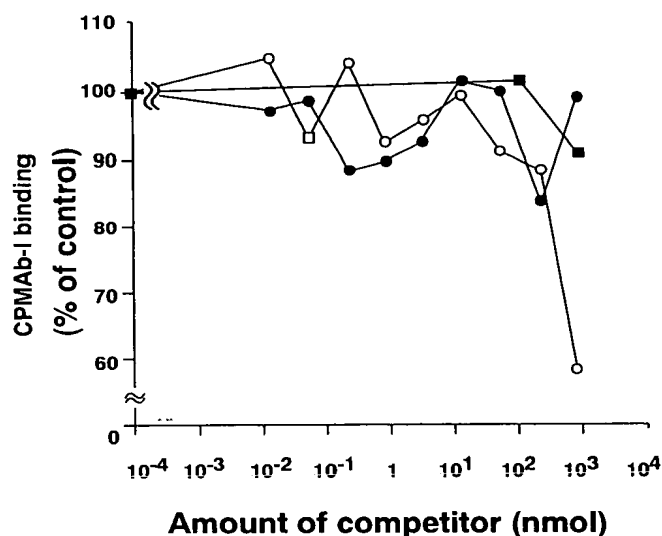


FIG. 2. Immunochemical specificity of the conformation-specific monoclonal antibody. The percentage of the antibody bound to the cCD-Multi-Pin-Block was determined in the presence and absence of competitors. Effectors used were: cCD-MAP (○), linear CD-MAP (●), and MAP protected with glycidol (■, amino groups of MAP were treated with excess amounts of 1,2-epoxy glycidol). Each point represents the mean of two determinations; nmol is expressed as nanomol per ELISA system.

Antiviral Activity

The anti-HIV-1 activities of CPMab-I were determined using MAGIC-5 cells (19). The cells were separately inoculated with various strains of HIV-1 (X4 virus, LAV-1_{BRU}; R5 virus, JRFL; R5X4 virus, 89.6 (23); and a clinical isolate, KMT strain (24), in the presence or absence of the antibody (twofold serial dilution). The number of infected (blue) cells was determined microscopically. The antibody, CPMab-I, markedly suppressed acute infection of MAGIC-5 cells by LAV-1_{BRU}, JRFL, 89.6, or KMT (clinical isolate) in a dose-dependent manner (Fig. 4).

Chemokine Signaling in the Presence of CPMab-I

The cocultivation was carried out in the transwell to show whether CPMab-I inhibited HIV-1 infection via CXCR4 without affecting the CXCR4 signaling responsible for cellular functions because CXCR4(+) cells may be exposed to the antibody raised against the cCD-MAP continuously rather than transiently *in vivo*. Therefore, the suppression of HIV-1 infection with CPMab-I was determined by cocultivation of Molt4#8 cells (lower chamber) and Hyb-I or Hyb-p2 (upper chamber) in the transwell (Fig. 5A). Molt4#8 cells were incubated for 24 h in the presence of Hyb-I or Hyb-p2 (control hybridoma producing isotype IgM κ), infected with HIV-1, further incubated at 37°C for 72 h with Hyb-I or Hyb-p2, and harvested. HIV-1 proviral DNA

was detected by PCR analysis. As shown in Fig. 5A, no HIV-1 proviral DNA was detectable in the cocultivation of Molt4#8 cells infected with HIV-1 and Hyb-I producing CPMab-I (lane 3), while either in the absence of Hyb-I (lane 2) or in the presence of Hyb-p2 producing isotype IgM κ (lane 4) was ascertained. These results prove that CPMab-I actually inhibits HIV-1 infection. To determine whether CPMab-I affects the cell functions following the blockade of the CXCR4 receptor, the cell migration and calcium influx in Molt4#8 cells induced by a chemokine, SDF-1 α , were investigated. SDF-1 α alone induced cell migration and

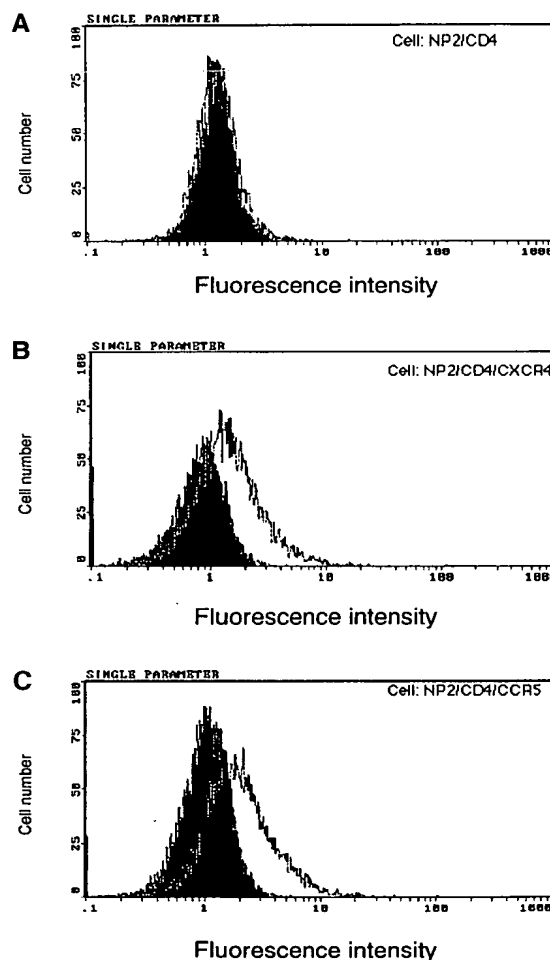
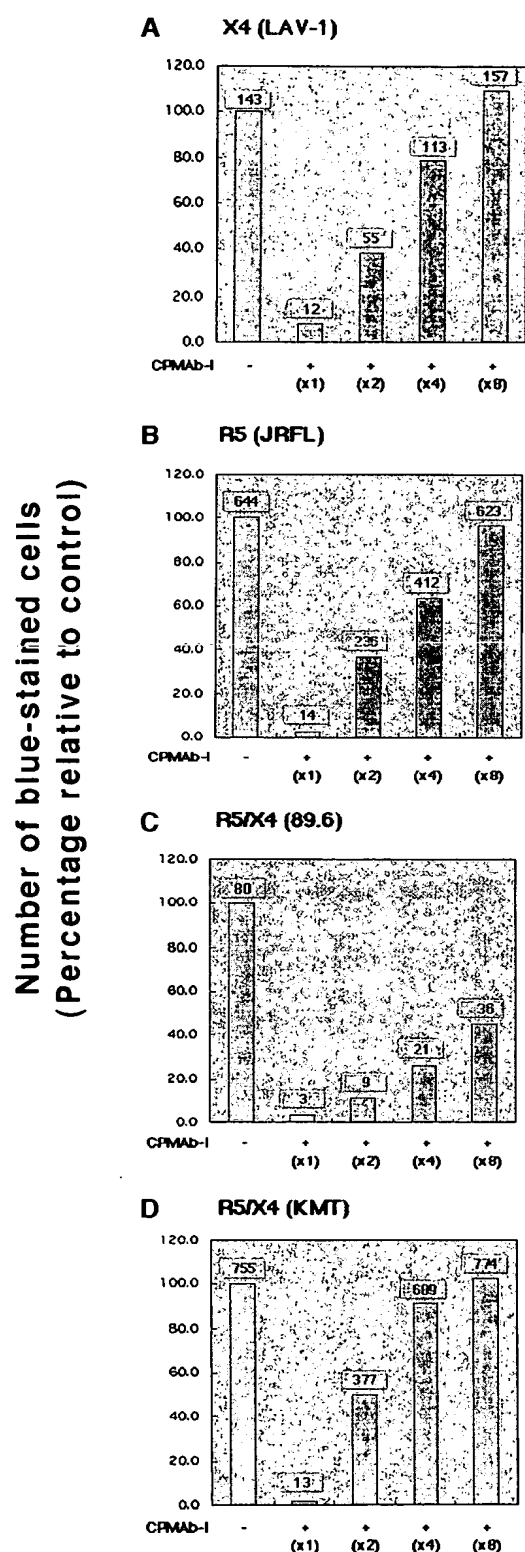


FIG. 3. Flow cytometry of NP-2 transfectants with CPMab-I. NP2/CD4 (A), NP2/CD4/CXCR4 (B), and NP2/CD4/CCR5 (C) were detached by incubation in PBS(-) containing 0.25% trypsin at 37°C for 1 min, then suspended in a cold washing buffer (PBS containing 2% FCS and 0.02% NaN₃) at 1×10^6 cells/ml. These were subjected to flow cytometry as described under Materials and Methods in which the cells were separately incubated with CPMab-I (1 μ g, solid line in Figs. 3A–3C), isotype-matched IgM antibody (control: 1 μ g, gray area in Figs. 3A–3C) at 4°C. Then, the cells were washed with the washing buffer and then resuspended in the washing buffer containing FITC-conjugated anti-mouse IgM.



Twofold serial dilution of the antibody-containing medium

calcium influx in Molt4#8 cells (Figs. 5B and 5C). The SDF-1 α -induced chemotaxis was not significantly disrupted in Molt4#8 cells cocultured with Hyb-1 for 96 h as well as with Hyb-p2 (Fig. 5B). Moreover, 12G5 significantly inhibit SDF-1 α -induced calcium influx (Fig. 5E), while CPMab-I was ineffective in the same assay (Fig. 5D). These results indicate that CPMab-I does not significantly interfere with calcium influx or chemotaxis induced by SDF-1 α . Taken together, the results indicate that CPMab-I inhibits HIV-1 infection via CXCR4, without affecting the CXCR4 signaling responsible for cellular functions.

DISCUSSION

It is generally thought that the conformational B-cell epitopes involved in inducing conformation-specific antibodies would be difficult to mimic using a simple synthetic linear peptide. Therefore, we attempted to develop an antigen providing a three-dimensional conformation in the peptide structure using MAP. The linear side-chain blocked oligopeptide with a free-amino terminal head and carboxyl terminal tail was first synthesized, and then cyclized by peptidyl bond formation between the α -amino group of Asp₁ and the α -carboxyl group of Gly₁₂ on the basis of the deduced conformation of the critical domain for HIV-1 entry (UPA of ECL-2) in CCR5 and CXCR4 structures. After removing the blocking groups of the linear CD and cCD, their molecular masses were determined by MALDI-TOF MS, in which case, the molecular mass of 18.36 was subtracted from that of the linear CD, and the difference in molecular mass between the linear CD and cCD indicates the formation of a peptide bond. The cCD was eluted with a slightly increased retention time compared with the corresponding linear CD (data not shown). On the basis of these results, it was established that the structure of cCD is DSQKEGEADDRG.

Five residues, SQKEG of cCD, derived from UPA of ECL2 in CCR5 have been highly conserved in humans (SWISS-PROT P51681; residues 169–173), green monkeys (SWISS-PROT P56493; residues 169–173), chimpanzees (SWISS-PROT P56440; residues 169–173), and mice (SWISS-PROT P51682; residues 171–175). On the other hand, the amino acid sequence, EADDR of cCD, derived from UPA of human CXCR4 shows 80% identity with those of the crab-eating macaque

FIG. 4. Antiviral activities. MAGIC-5 cells were separately incubated with various HIV-1 strains (X4 virus, LAV-1_{BRU} A, amount of HIV-1 p24 antigen, 56 ng; R5 virus, JRFL B, 5.6 ng; R5X4 virus, 89.6 C, 179 ng; and KMT (clinical isolate) D, 280 ng, and cocultured with the antibody-containing medium (400 μ l, CPMab-I: A, B, C, D) for 48 h. No significant cytotoxicity of the antibody-containing medium was observed. Values on the top of the column denote the number of blue-stained cells and represent the mean of triplicate determinations.

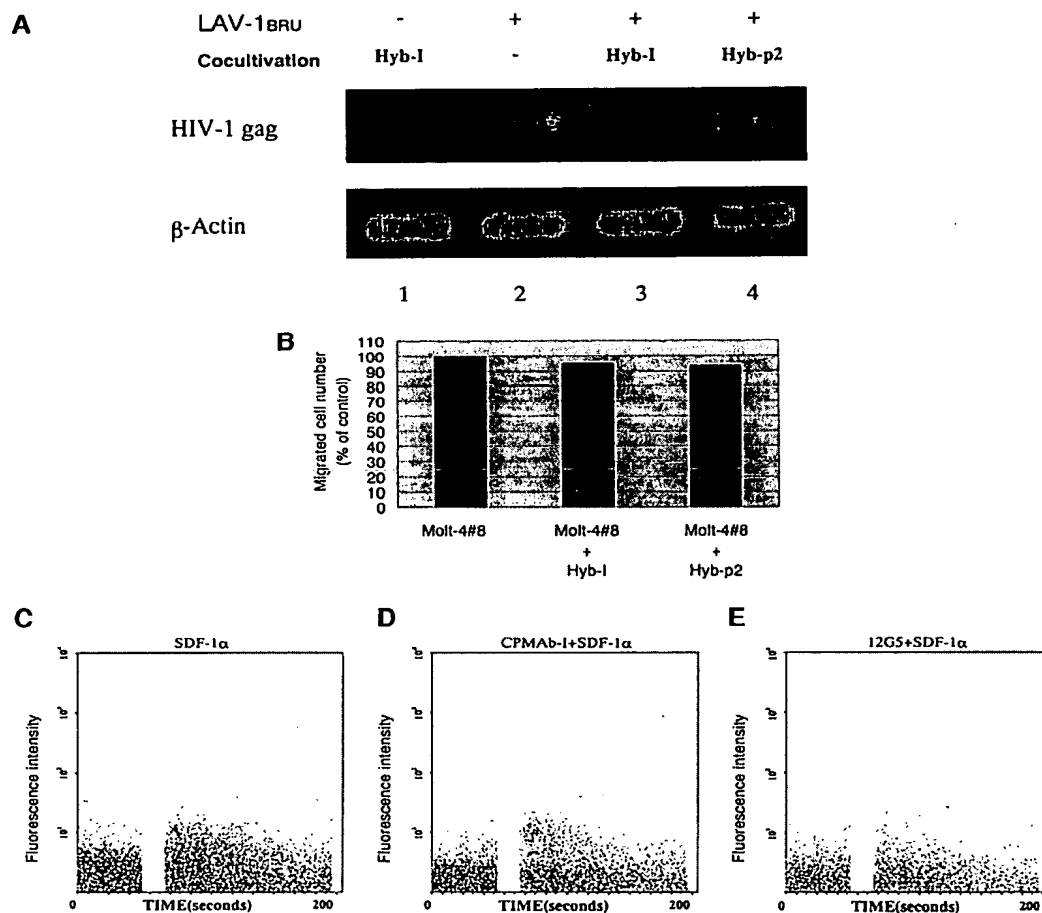


FIG. 5. Effect of CPMab-I on X4 HIV-1 replication and chemokine signaling. (A) After 24 h of coculture, Molt4#8 cells were infected with HIV-1 (m.o.i. = 0.01) or inoculated into the RPMI1640 medium, and then incubated with Hyb-I or Hyb-p2 at 37°C for an additional 72 h. Molt4#8 cells were harvested, and the detection of HIV-1 proviral DNA was carried out by PCR analysis as described under Materials and Methods. (B) SDF-1 α -induced Molt4#8 migration was not significantly affected by coculture with Hyb-I. (C) SDF-1 α was added to the Fluo-3, AM-loaded Molt4#8 cells pretreated anti-CXCR4 mAb, 12G5, and CPMab-I for 24 h. Changes in intracellular calcium concentration in response to SDF-1 α were measured with EPICS XL.

(SWISS-PROT Q28474; residues 179–183) and rhesus macaque (SWISS-PROT P79394; residues 179–183), and 60% identity with that of the mouse (SWISS-PROT P70658; residues 186–190).

The binding specificity of the antibodies against cyclic chimeric dodecapeptide antigen was first determined using a flow cytometer. The immunoreaction was affected by cCD even in ELISA using the cCD-Multi-Pin Block, but not by the linear CD. The antibody, CPMab-I, evidently reacted with both the NP2/CD4/CCR5 and NP2/CD4/CXCR4 cells, but not with the nonCCR5- and nonCXCR4-expressing cells. Thus, these results taken together indicate that the monoclonal antibody against cCD-MAP is conformation-specific, and it recognizes the conformation-specific and critical domain of UPA of ECL-2 in CCR5 or CXCR4.

Thus, the anti-HIV-1 activities of CPMab-I were determined using MAGIC-5 cells which express CCR5. The cells were inoculated separately with various strains of HIV-1 (X4, R5, and R5X4) in the presence or absence of the antibody (twofold serial dilution). As expected, CPMab-I markedly suppressed infection by various HIV-1 strains. To determine whether the antibody affects the cell functions following the blockade of the chemokine receptor, the calcium influx and chemotaxis induced by SDF-1 α was also investigated using Molt4#8 cells. The antibody did not significantly interfere with calcium influx or chemotaxis induced by SDF-1 α .

Several vaccines based on native or recombinant viral materials have so far failed to provide protection against heterologous virus infection in regard to strategies used for the production of AIDS vaccines. There

is, therefore, an urgent need for an effective vaccine against HIV-1.

Recent studies have indicated that the CCR5 extracellular domains involved in chemokine ligand specificity and in coreceptor usage of various HIV-1 strains are not identical (25). Moreover, the amino terminal and ECL-2 of CCR5 have been implicated in coreceptor functions, while the second extracellular loop is the major determinant of ligand specificity (26). Olson *et al.* demonstrated that there is no correlation between the ability of a monoclonal antibody to inhibit HIV-1 fusion-entry and its ability to inhibit either the binding of a gp120-soluble CD4 complex to CCR5 or CC-chemokine activity (15). Therefore, the development of a new immunotherapy, which does not interfere with chemokine binding yet blocks viral infection, is indispensable for AIDS therapy in the near future.

Here, we show evidence for the potential use of an HIV-1-coreceptor-based defense vaccine, which was synthesized focusing on the potential and conformation-specific site of a chemokine receptor responsible for HIV-1 infection. Both the native UPAs (Asn₁₇₆ to Cys₁₈₆ in ECL-2 of CXCR4 and Arg₁₆₈ to Cys₁₇₈ in ECL-2 of CCR5) are shown to be conformation-specific, and likely to form small cyclic portions because of the formation of a disulfide bond between Cys₁₈₆ and Cys₁₀₉ in ECL-1 of CXCR4 or between Cys₁₇₈ and Cys₁₀₁ in ECL-1 of CCR5. Therefore, our studies demonstrate that a cCD mimicking the UPA of ECL-2 can be constructed by cyclization of both the pentapeptides, S₁₆₉-Q₁₇₀-K₁₇₁-E₁₇₂-G₁₇₃ of CCR5 and E₁₇₉-A₁₈₀-D₁₈₁-D₁₈₂-R₁₈₃ of CXCR4, and the spacer-armed Gly-Asp dipeptide. The cCD-MAP can induce autoantibodies that cross-react with both CCR5 and CXCR4, as determined by flow cytometry, block HIV-1 infection (X4, R5, and R5X4 HIV-1) in MAGIC-5 assay, and also prevent LAV-1_{BRU} infection by specifically targeting CXCR4 on the cell surface of Molt4#8 cells cocultured with Hyb-I without affecting SDF-1 α -induced chemotaxis.

Thus, the antibodies raised against the cCD-MAP derived from the UPA of ECL-2 in CCR5 and CXCR4 will be potentially useful for protection or defense against HIV-1 infection, and the cCD-MAP or its derivative immunogen may be a novel HIV-1 vaccine.

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REFERENCES

1. Almond, N. M., and Heeney, J. L. (1998) AIDS vaccine development in primate models. *AIDS* **12**, 133–140.
2. Burton, D. R., and Moore, J. P. (1998) Why do we not have an HIV vaccine and how can we make one? *Nat. Med.* **4**, 495–498.
3. Burastero, S. E., Gaffi, D., Lopalco, L., Tambussi, G., Borgonovo, B., De, S. C., Abecasis, C., Robbioni, P., Gasparri, A., Lazzarin, A., Celada, F., Siccardi, A. G., and Beretta, A. (1996) Autoantibodies to CD4 in HIV type 1-exposed seronegative individuals. *AIDS Res. Hum. Retroviruses* **12**, 273–280.
4. Lopalco, L., Barassi, C., Pastori, C., Longhi, R., Burastero, S. E., Tambussi, G., Mazzotta, F., Lazzarin, A., Clerici, M., and Siccardi, A. G. (2000) CCR5-reactive antibodies in seronegative partners of HIV-seropositive individuals down-modulate surface CCR5 *in vivo* and neutralize the infectivity of R5 strains of HIV-1 *in vitro*. *J. Immunol.* **164**, 3426–3433.
5. Lopalco, L., Pastori, C., Cosma, A., Burastero, S. E., Capiluppi, B., Boeri, E., Beretta, A., Lazzarin, A., and Siccardi, A. G. (2000) Anti-cell antibodies in exposed seronegative individuals with HIV type 1-neutralizing activity. *AIDS Res. Hum. Retroviruses* **16**, 109–115.
6. Mosier, D. E., Picchio, G. R., Gulizia, R. J., Sabbe, R., Poignard, P., Picard, L., Offord, R. E., Thompson, D. A., and Wilken, J. (1999) Highly potent RANTES analogues either prevent CCR5-using human immunodeficiency virus type 1 infection *in vivo* or rapidly select for CXCR4-using variants. *J. Virol.* **73**, 3544–3550.
7. Este, J. A., Cabrera, C., Blanco, J., Gutierrez, A., Bridger, G., Henson, G., Clotet, B., Schols, D., and De, Clercq, E. (1999) Shift of clinical human immunodeficiency virus type 1 isolates from X4 to R5 and prevention of emergence of the syncytium-inducing phenotype by blockade of CXCR4. *J. Virol.* **73**, 5577–5585.
8. Stewart, G. (1998) Chemokine genes-beating the odds. *Nat. Med.* **4**, 275–277.
9. Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1996) CC CKR5: A RANTES, MIP-1 α , MIP-1 β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**, 1955–1958.
10. Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M. D., Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R. (1996) Identification of a major coreceptor for primary isolates of HIV-1. *Nature* **381**, 661–666.
11. Doranz, B. J., Orsini, M. J., Turner, J. D., Hoffman, T. L., Berson, J. F., Hoxie, J. A., Peiper, S. C., Brass, L. F., and Doms, R. W. (1999) Identification of CXCR4 domains that support coreceptor and chemokine receptor functions. *J. Virol.* **73**, 2752–2761.
12. Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P., and Paxton, W. A. (1996) HIV-1 entry into CD4⁺ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667–673.
13. Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872–877.
14. Lee, B., Sharron, M., Blanpain, C., Doranz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Guy, H. R., Durell, S. R., Parmentier, M., Chang, C. N., Price, K., Tsang, M., and Doms, R. W. (1999) Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. *J. Biol. Chem.* **274**, 9617–9626.
15. Olson, W. C., Rabut, G. E., Nagashima, K. A., Tran, D. N., Anselma, D. J., Monard, S. P., Segal, J. P., Thompson, D. A., Kajumo, F., Guo, Y., Moore, J. P., Maddon, P. J., and Dragic, T. (1999) Differential inhibition of human immunodeficiency virus type 1 fusion, gp120 binding, and CC-chemokine activity by monoclonal antibodies to CCR5. *J. Virol.* **73**, 4145–4155.

16. Rucker, J., Samson, M., Doranz, B. J., Libert, F., Berson, J. F., Yi, Y., Smyth, R. J., Collman, R. G., Broder, C. C., Vassart, G., Doms, R. W., and Parmentier, M. (1996) Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. *Cell* **87**, 437–446.
17. Siciliano, S. J., Kuhmann, S. E., Weng, Y., Madani, N., Springer, M. S., Lineberger, J. E., Danzeisen, R., Miller, M. D., Kavanaugh, M. P., DeMartino, J. A., and Kabat, D. (1999) A critical site in the core of the CCR5 chemokine receptor required for binding and infectivity of human immunodeficiency virus type 1. *J. Biol. Chem.* **274**, 1905–1913.
18. Soda, Y., Shimizu, N., Jinno, A., Liu, H.-Y., Kanbe, K., Kitamura, T., and Hoshino, H. (1999) Establishment of a new system for determination of coreceptor usages of HIV based on the human glioma NP-2 cell line. *Biochem. Biophys. Res. Commun.* **258**, 313–321, doi: 10.1006/bbrc.1999.0633.
19. Hachiya, A., Aizawa-Matsuoka, S., Tanaka, M., Takahashi, Y., Ida, S., Gatanaga, H., Hirabayashi, Y., Kojima, A., Tatsumi, M., and Oka, S. (2001) Rapid and simple phenotypic assay for drug susceptibility of human immunodeficiency virus type 1 using ccr5-expressing hela/cd4(+) cell clone 1-10 (magic-5). *Antimicrob. Agents Chemother.* **45**, 495–501.
20. Zack, J. A., Arrigo, S. J., Weitsman, S. R., Go, A. S., Haislip, A., and Chen, I. S. Y. (1990) HIV-1 entry into quiescent primary lymphocytes: Molecular analysis reveals a labile, latent viral structure. *Cell* **61**, 213–222.
21. Gosling, J., Monteclaro, F. S., Atchison, R. E., Arai, H., Tsou, C. L., Goldsmith, M. A., and Charo, I. F. (1997) Molecular uncoupling of C-C chemokine receptor 5-induced chemotaxis and signal transduction from HIV-1 coreceptor activity. *Proc. Natl. Acad. Sci. USA* **94**, 5061–5066.
22. Ido, Y., Misumi, S., Furuishi, K., and Shoji, S. (1998) 15th Japan Pharmaceutical Sciences Kyushu Branch Meeting in Fukuoka. Eds. by Suemune, H. Abstract, pp. 43.
23. Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G., and Doms, R. W. (1996) A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**, 1149–1158.
24. Yoshimura, K., Matsushita, S., Hayashi, A., and Takatsuki, K. (1996) Relationship of HIV-1 envelope V2 and V3 sequences of the primary isolates to the viral phenotype. *Microbiol. Immunol.* **40**, 277–287.
25. Wu, L., LaRosa, G., Kassam, N., Gordon, C. J., Heath, H., Ruffing, N., Chen, H., Humblies, J., Samson, M., Parmentier, M., Moore, J. P., and Mackay, C. R. (1997) Interaction of chemokine receptor CCR5 with its ligands: Multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. *J. Exp. Med.* **186**, 1373–1381.
26. Samson, M., LaRosa, G., Libert, F., Paindavoine, P., Detheux, M., Vassart, G., and Parmentier, M. (1997) The second extracellular loop of CCR5 is the major determinant of ligand specificity. *J. Biol. Chem.* **272**, 24934–24941.

A Novel Cyclic Peptide Immunization Strategy for Preventing HIV-1/AIDS Infection and Progression*

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A novel synthetic peptide immunogen targeting the human immunodeficiency virus type-1 (HIV-1) coreceptor CXCR4 was evaluated for its capacity to induce CXCR4-specific antibodies with anti-HIV-1 activity in BALB/c mice and cynomolgus monkeys. A cyclic closed-chain dodecapeptide mimicking the conformation-specific domain of CXCR4 (cDDX4) was prepared in which Gly-Asp, as the dipeptide forming a spacer arm, links the amino and carboxyl termini of the decapeptidyl linear chain (linear DDX4, Asn¹⁷⁶ to Ile¹⁸⁵) derived from the undcapeptidyl arch (UPA; Asn¹⁷⁶ to Cys¹⁸⁶) of extracellular loop 2 (ECL-2) in CXCR4. Immunization of BALB/c mice with cDDX4 conjugated with a multiple-antigen peptide (cDDX4-MAP) induced conformational epitope-specific antibodies, and monoclonal antibody IA2-F9 reacted with cDDX4, but not with linear DDX4, as determined by real-time biomolecular interaction analysis using surface plasmon resonance. The antibody also reacted with cells expressing CXCR4 but not with cells expressing the other HIV coreceptor, CCR5. Furthermore, the antibody inhibited the replication of HIV-1 X4 virus (using CXCR4), as shown by an infection assay using both MAGIC-5 cells and MT4 cells, but not that of HIV-1 R5 virus (using CCR5). The antibody weakly interfered with chemotaxis induced by stromal cell-derived factor-1 α in THP-1 cells or moderately inhibited the chemotaxis of Molt4#8 cells under the same conditions. In addition, immunization of cynomolgus monkeys also induced cDDX4-specific antibodies with anti-HIV activity. Taken together, these results indicate that cDDX4 conjugated with a multi-antigen peptide induces the conformational epitope-specific antibodies to the undcapeptidyl arch of CXCR4 may be a novel candidate immunogen for preventing disease progression in HIV-1-infected individuals.

Human immunodeficiency virus type-1 (HIV-1)¹ requires both CD4 and a chemokine receptor for cellular entry. Follow-

ing its binding to CD4, the viral envelope protein changes its conformation to bind to the chemokine receptor and initiates fusion with the cellular membrane (1–4). Because chemokine receptors CCR5 and CXCR4 are the main coreceptors for cellular entry of HIV-1, viral strains are classified as R5, X4, or R5X4 according to the usage of chemokine receptor (5, 6). HIV-1 R5 virus generally transmits the infection, and predominates in an early stage of infection and in long-term nonprogressors (1, 4). In contrast, HIV-1 X4 virus emerges during chronic infection, and its emergence is associated with a rapid decline in the count of CD4 (+) T cells and progression to AIDS (7–9). The highly active antiretroviral therapy (HAART) has a marked effect on the epidemic of AIDS, whereas the limitations of HAART, such as the emergence of drug-resistant HIV-1 variants and several inherent adverse effects, were also reported (10–12). Therefore, development of an antiviral drug that targets novel molecules is desirable (13). CXCR4 antagonists that prevent entry of X4 virus are one of the candidates. Some studies showed that blockade of CXCR4 can possibly prevent emergence of X4 virus and change the phenotype of already existing X4 virus to R5 virus (14, 15). In addition, a recent statistical study showed that long term nonprogressors have high levels of plasma stromal cell-derived factor-1 α (SDF-1 α), which is a ligand for CXCR4, and low CXCR4 expression levels on T lymphocytes; in advancing disease, the expression level of CXCR4 increases (16). These reports indicate that CXCR4 is an attractive target, which not only inhibits the entry of X4 virus but may also delay the disease progression to AIDS, and may become the target of immunotherapeutic approaches.

CXCR4 has been shown to be critical for development (17–19) but is probably dispensable in adults. However, when developing a self-antigen as a target of an immunotherapeutic approach, unexpected effects must be taken into consideration together with sufficient immune responses. Hence, it is desirable to design an immunogen that targets a restricted protein region rather than the whole protein. Therefore, a linear peptide immunogen has the advantage in that it can elicit a desired immune response against a restricted protein region, whereas it can hardly induce antibodies against the conformational epitope (20, 21). Indeed, because CXCR4 is a member of the seven transmembrane-spanning G protein-coupled receptor family, and it has conformational and flexible extracellular

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¹ The abbreviations used are: HIV, human immunodeficiency virus; cDDX4, cyclic closed-chain dodecapeptide mimicking the conformation-specific domain of CXCR4; cDDX4-MAP, cDDX4 conjugated with a multiple-antigen peptide; ECL, extracellular loop; HAART, highly active antiretroviral therapy; UPA, undcapeptidyl arch; SDF-1 α , stromal

cell-derived factor-1 α ; PBMCs, peripheral blood mononuclear cells; mAb, monoclonal antibody; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; MOE, molecular operating environment; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; MAP, multiple-antigen peptide; FCS, fetal calf serum; PBS, phosphate-buffered saline; m.o.i., multiplicity of infection.

regions, most conformational-specific anti-CXCR4 antibodies were induced by direct immunization of cells that expressed human CXCR4 (22, 23). Therefore, the antibody induced by a linear synthetic peptide against the extracellular loop region of CXCR4 could recognize a denatured protein but could not recognize the native protein. A linear peptide is inadequate for mimicking the conformational loop region of CXCR4, and mimicking the native conformational epitope by a peptide is critical for inducing the antibody against a conformational epitope. Therefore, to use the peptide immunogen for an immunotherapeutic approach targeting CXCR4, it is essential for the designed peptide immunogen to target not only the restricted region of CXCR4 but also to mimic the conformational epitope of the targeted region.

In this study, we investigated the application of a cyclic dodecapeptide peptide (cDDX4) that was designed to mimic the native conformational epitope of the undeca-peptidyl arch (UPA) (24, 25) in CXCR4 for use as a novel immunotherapy for AIDS. Immunization with cDDX4 conjugated with a multiantigen peptide (cDDX4-MAP) induced conformational epitope-specific antibodies that preferentially recognized the cyclic structure of the antigen and cross-reacted with cell surface CXCR4. In addition, the induced antibody inhibited the replication of HIV-1 X4 virus but not that of HIV-1 R5 virus as determined by infection assay using MAGIC-5 cells and by a productive infection assay using MT4 cells. These results indicate that cDDX4 sufficiently mimics the conformational epitope of UPA in cell surface CXCR4 and possibly induces antibodies that inhibit cellular entry of HIV-1. In addition, immunization of cynomolgus monkeys with cDDX4-MAP also elicited cDDX4-specific antibodies with anti-HIV activity. Taken together, we propose that an immunotherapeutic approach using cDDX4-MAP as the self-antigen immunogen may be a novel strategy for AIDS therapy.

EXPERIMENTAL PROCEDURES

Cell Culture—The human T-lymphotropic virus type I-infected cell line (MT4), human T cell lines (CEM and Molt4#8), human monocytic cell lines (THP-1 and U937), and peripheral blood mononuclear cells (PBMCs) were cultured at 37 °C in 5% (v/v) CO₂ in the RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). MAGIC-5 cells and CD4-transduced human glioma cell lines (NP2/CD4, NP2/CD4/CXCR4, and NP2/CD4/CCR5) were cultured at 37 °C in 5% (v/v) CO₂ in Dulbecco's modified Eagle's medium supplemented with 2.5% or 10% FCS, respectively.

Anti-CXCR4 Antibody Generation—The antigen and antibody were prepared using the protocol of Misumi *et al.* (24). To mimic the native conformational epitope of human CXCR4, the CXCR4-derived linear dodecapeptide (linear DDX4: N₂H-DNVSEADDDRYIG-COOH) was synthesized using an automatic peptide synthesizer and then cyclized. The cyclic dodecapeptide (cDDX4: DNVSEADDDRYIG) was conjugated with a multiple-antigen peptide (MAP) through the formation of the peptide bond between the β -carboxyl group of Asp within the cDDX4 and the amino group within MAP. MAP, which is composed of 2-fold bifurcating polylysine core developed as a carrier of a peptide antigen, is capable of eliciting a strong antibody response in mice, monkeys, and humans (25). All peptides were purified by high-performance liquid chromatography (Waters), and their molecular masses were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Burkert Franzen Analytik GmbH, Bremen, Germany).

Female BALB/c mice were immunized with the cDDX4-MAP conjugate using the protocol of Galfre and Milstein (26). The resulting hybridoma that produced the most potent supernatant was screened in 96-well plates for the reactivity to Multi-Pin Block (Chiron Technologies), which was conjugated with cDDX4 and was cloned by limiting dilution. The anti-CXCR4 antibody, IA2-F9, was recovered by ammonium sulfate precipitation and purified using a Sephadex G-150 column (Amersham Biosciences).

Real-time Biomolecular Interaction Analysis using Surface Plasmon Resonance—Recognition of the cyclic structure of cDDX4 by IA2-F9 was analyzed by the surface plasmon response technology using BIAcore2000 as described previously (24, 27, 28). The free β -carboxyl group

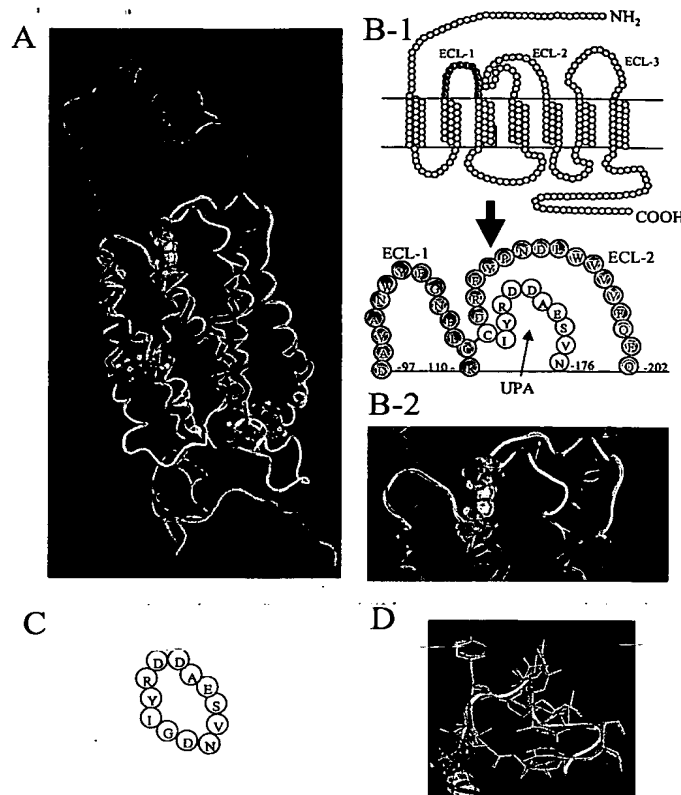


FIG. 1. Deduced structure of UPA in ECL-2 of CXCR4. A, predicted model of CXCR4 was constructed using the segmented approach, and MOE was used for actual calculation. The determined structure of rhodopsin was used as the template (30). Transmembrane helices are indicated in cyan. The extracellular loops of CXCR4 are color-coded: amino terminus, blue; ECL-1, green; ECL-2, yellow and orange; UPA, yellow; and ECL-3, magenta. The intercellular loops are indicated in white and the C terminus in red. B, predicted model of UPA in CXCR4. Because cysteine residues in ECL-1 and ECL-2 form a disulfide bond, the ECL-2 region of CXCR4 has a unique arch structure consisting of 11 amino acid residues (UPA, undeca-peptidyl arch, yellow). In this study, UPA was selected as the target of peptide immunogen. C and D, to mimic the native conformational epitope of UPA in CXCR4, the dodecapeptide (¹⁷⁶DNVSEADDDRYI¹⁸⁵) derived from the UPA sequence was cyclized by insertion of the spacer-armed dipeptide (Gly-Asp, red), and the deduced structure of cDDX4 (shown in blue) was adopted to the structural model of UPA in CXCR4 using the MOE-Align tool.

of Asp that was used as a spacer-armed dipeptide in cDDX4 was conjugated to 5-[5-(N-succinimidylloxycarbonyl)pentylamido]hexyl D-biotinamide through ethylenediamine. Biotinylated cDDX4 was immobilized to streptavidin-coated sensor chips. For competition assay, purified IA2-F9 was pretreated with cDDX4 (1 and 10 nmol) or linear DDX4 (1 and 10 nmol) at room temperature prior to BIAcore analysis. All the antigen-antibody interactions were analyzed in a binding buffer (0.02% KH₂PO₄, 0.29% Na₂HPO₄, 12H₂O, 0.8% NaCl, and 0.02% KCl) at a constant flow rate of 50 μ l/min and a constant temperature of 25 °C. The bound antibody was eluted from the biotinylated cDDX4 by a short pulse (20 μ l) of 10 mM Gly-HCl (pH 2.0). This regeneration procedure did not alter the ability of cDDX4 to bind to the antibody in subsequent cycles. Kinetic analysis was performed on BIAcore2000 using BIAcore evaluation software. For detecting anti-cDDX4 antibodies in sera derived from immunized cynomolgus monkeys, the sera were dialyzed using Spectra/Por (cutoff molecular masses, 100,000; Spectrum Laboratories Inc.) according to the manufacturer's instructions and analyzed.

Antibody and Flow Cytometry—The following antibodies were used: an anti-CXCR4 mAb (clone 12G5, BD Biosciences), an isotype-matched control antibody (Sigma), purified IA2-F9, and fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G or anti-mouse IgM. Cells (1 \times 10⁶) were washed with washing buffer (PBS containing 2% FCS and 0.02% NaN₃) and incubated with 10 μ g/ml primary antibody (purified IA2-F9 or 12G5) for 30 min at 4 °C. The cells were then

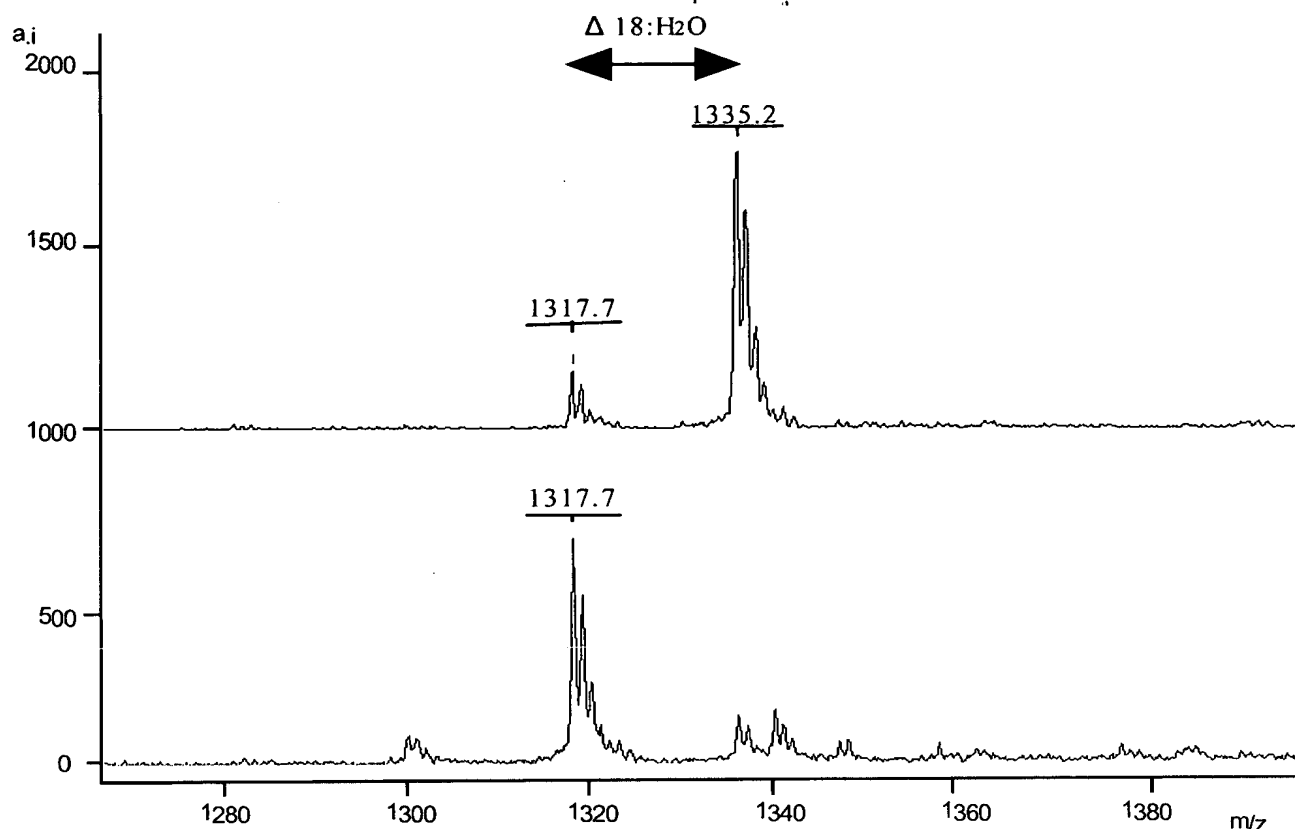


FIG. 2. MALDI-TOF MS spectra of linear DDX4 and cDDX4. The spectra exhibit two peaks at m/z 1317.7 and 1335.2: the upper peak corresponds to the ion derived from linear DDX4, and the lower peak to the ion derived from cDDX4. The matrix was a saturated solution of α -cyano-4-hydroxycinnamic acid in solution of acetonitrile-water (1:2, v/v) containing 0.1% trifluoroacetic acid. The fraction with a molecular mass of 17.5 corresponding to H_2O was deleted after cyclizing the linear DDX4 with the peptide bond.

washed with the same washing buffer and incubated with FITC-conjugated anti-mouse IgG or anti-mouse IgM. Then the cells were washed again and analyzed using an EPICS XL flow cytometer (Beckman Coulter).

Chemotaxis Assay—The migration of Molt4#8 and THP-1 was assayed in 24-well cell-culture chambers using an insert with 5.0- μ m pore membranes (Corning, Corning, NY) according to the protocol of Gosling *et al.* (29) with slight modifications. Cells (5×10^6) were pretreated with or without IA2-F9 for 30 min and were placed in the upper chamber. Chemotaxis was conducted in the presence of 10 nM SDF-1 α (placed in the lower chamber). After incubation for 3 h at 37 °C, cells that migrated from the upper chamber to the lower chamber were quantified by trypan blue dye exclusion.

MAGIC-5 Assay—The antiviral activity of IA2-F9 was determined by an infection assay using MAGIC-5 cells (32). Because MAGIC-5 cells express CD4, CCR5, and CXCR4, they are susceptible to infection by R5, X4, and R5X4 viruses. The cells were seeded (1×10^4 cells per well) and cultured in a 48-well plate for 24 h. After removal of the medium from each well, the cells were incubated for 30 min with IA2-F9 at the indicated concentrations and were infected with the virus in the presence of DEAE-dextran (20 μ g/ml) for 2 h and washed with the culture medium. The cells were cultured in the medium containing IA2-F9 for 48 h, fixed with 1% formaldehyde-0.2% glutaraldehyde in PBS for 5 min, washed, and then stained with X-gal. The number of blue-stained cells was counted under a light microscope. Control experiments were carried out under identical conditions in the absence of the antibody. For determining anti-HIV activity of sera at 0 and 10 weeks derived from immunized cynomolgus monkeys, antibodies were purified from the serum using an NAbTM Protein L Spin kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. For determining anti-HIV activity of sera at 25 and 27 weeks, sera were dialyzed with PBS(–) using Spectra/Por (cutoff molecular mass, 100 kDa; Spectrum Laboratories Inc.) and adjusted to a concentration corresponding to a 1/10 dilution of sera with PBS(–).

Productive Infection Assay—MT4 cells (1×10^5) were preincubated

with IA2-F9 at the indicated concentrations for 30 min, and then HIV-1 X4 virus (m.o.i. = 0.01) was inoculated to these cells. After washing three times with PBS, the cells were incubated with IA2-F9 (0, 1, and 10 μ g/ml) for 72, 96, and 120 h. Then the culture supernatant of each cell was collected, and the p24 antigen level was measured by antigen-capture enzyme-linked assay using a RETRO-TEK HIV-1 p24 antigen enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp.) according to the manufacturer's instructions.

Immunization Schedule—All the cynomolgus monkeys were housed in individual cages and maintained according to the rules and guidelines of the National Institute for Infectious Diseases for experimental animal welfare. Three cynomolgus monkeys, 4–6 years old, were immunized intraperitoneally at 0 and 1 week with 300 μ g of cDDX4-MAP in complete Freund's adjuvant and boosted subcutaneously at 6 weeks with 300 μ g of cDDX4-MAP in incomplete Freund's adjuvant. Furthermore, these monkeys were reboosted subcutaneously at 25 weeks with 300 μ g of cDDX4-MAP in Freund's incomplete adjuvant. Another three cynomolgus monkeys were immunized with MAP following the same immunization schedule. Blood samples were obtained at 0, 10, 25, and 27 weeks, which were then subjected to BIAcore analysis and MAGIC-5 assay.

RESULTS

Design and Synthesis of cDDX4—The hypothetical structure model of CXCR4 was based on its homology with rhodopsin (30), and energy-minimized with the Molecular Operating Environment, MOE (Chemical Computing Group Inc., Montreal, Quebec, Canada) (Fig. 1A). The extracellular loop-2 (ECL-2) region of CXCR4, and its structure deduced by MOE (Figs. 1B-1 and 2) has a unique arch structure consisting of 11 amino acid residues (UPA) on the basis of the Cys¹⁸⁶ residue bound to a Cys¹⁰⁹ residue of ECL-1 by a disulfide bond (Swiss-Prot P30991, Fig. 1B-1). The cDDX4 moiety designed to mimic the

native conformational epitope of UPA was generated by cyclization of a decapeptide ($^{176}\text{NVSEADDRYI}^{185}$) derived from the UPA sequence by insertion of a spacer-armed dipeptide (Gly-Asp) (shown in red in Fig. 1C). The deduced structure of cDDX4 (shown in blue) was adopted to the structural model of UPA in CXCR4 using the MOE-Align tool (Chemical Computing Group Inc., Montreal, Quebec, Canada, shown in yellow in Fig. 1D).

Cyclization of the linear peptide was confirmed by molecular mass analysis using MALDI-TOF-MS (Fig. 2). The spectra of linear DDX4 and cDDX4 exhibited major peaks at m/z 1335.2 and 1317.7, respectively. The difference in molecular mass between linear DDX4 and cDDX4 indicates the formation of a peptide bond. The cDDX4 was combined with MAP to enhance immunogenicity and then immunized to BALB/c mice.

Conformational Specificity of IA2-F9—Immunization of BALB/c mice with cDDX4-MAP induced production of high titers of antibodies. Using cDDX4-Multi-Pin Block for a screening antigen, monoclonal antibody IA2-F9 that has the highest enzyme-linked immunosorbent assay titer was established. To analyze the conformational epitope-specific recognition of IA2-F9, the surface plasmon resonance technology using BIAcore2000 was applied. cDDX4 was immobilized to a sensor chip by streptavidin-biotin interaction. IA2-F9 recognized cDDX4 on the sensor chip in a dose-dependent manner ($K_d = 1.55 \times 10^{-8}$ M) (Fig. 3A). IA2-F9 binding competition was observed in the case of pretreatment of IA2-F9 with cDDX4 (Fig. 3B). In contrast, pretreatment of the linear peptide did not affect the binding of IA2-F9 (Fig. 3C). These results indicate that IA2-F9 is a conformational epitope-specific mAb that can recognize the cyclic structure of the cDDX4 antigen.

Binding of IA2-F9 to Cell Surface CXCR4—Mimicry of a conformational epitope by a peptide is critical for induction of antibodies that can recognize the native protein by a peptide antigen. Paradoxically, only a peptide antigen that mimics the conformational epitope could induce antibodies that react with the native protein. The binding of IA2-F9 to cells expressing CXCR4 was determined by flow cytometry. IA2-F9 could recognize CXCR4 but not CCR5 expressed on NP2/CD4 cells (Fig. 4, A–C). These results indicate that IA2-F9 is a conformational epitope-specific mAb and specifically recognizes cell surface CXCR4. Furthermore, analysis of a panel of cells demonstrated that all cells known to express CXCR4, including MT4, THP-1, MAGIC-5, Molt4#8, U937, and PBMCs, were stained by IA2-F9 (Fig. 4, D–I).

Chemotaxis Assay—The effect of IA2-F9 binding on the chemokine receptor activity was investigated by chemotaxis assay using SDF-1 α as the chemoattractant. Both human T cell lines Molt4#8 and THP-1 were used with or without IA2-F9. Although each cell line showed different chemotactic reactivities against SDF-1 α , IA2-F9 inhibited weakly (THP-1) or moderately (Molt4#8) the SDF-1 α -induced chemotaxis (Fig. 5).

Anti-HIV Activity of IA2-F9—Because ECL-2 is thought to be critical for HIV-1 X4 viral entry (31), anti-UPA antibodies are expected to inhibit the viral infection. The anti-HIV activity of IA2-F9 was determined by an infection assay using MAGIC-5 cells (32). MAGIC-5 cells were generated from CD4- and CXCR4-positive HeLa-CD4-LTR- β -gal (MAGI) cells by transfection with a CCR5 expression plasmid to confer susceptibility to infection of not only X4 virus but also R5 virus. MAGIC-5 cells were inoculated with LAV-1 (X4 virus), JRFL (R5 virus), or 89.6 (R5X4 virus), in the absence or presence of IA2-F9 at indicated concentrations. IA2-F9 inhibited infection of LAV-1 but not those of JRFL and 89.6 (Fig. 6).

To verify the antiviral activity of IA2-F9 in an alternative experiment, the inhibitory effect of IA2-F9 on HIV-1 replication in T cells was investigated. MT4 cells, a human T cell line, were

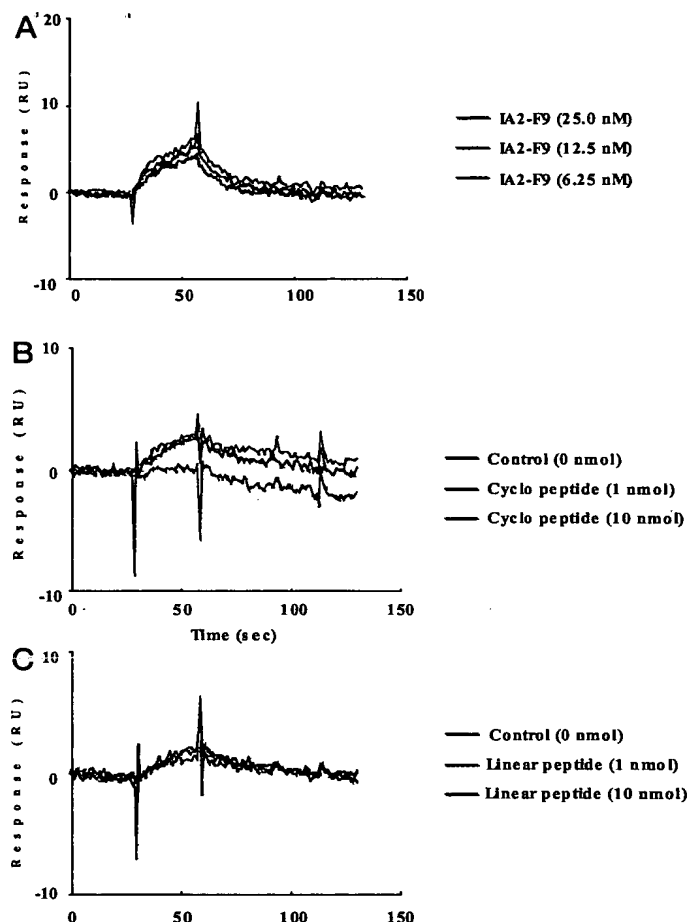


FIG. 3. Conformational specificity of IA2-F9. Recognition of the cyclic structure of cDDX4 by IA2-F9 was analyzed by surface plasmon response technology using BIAcore2000. Aliquots of the biotinylated cDDX4 were immobilized to the streptavidin-coated sensor chip and IA2-F9 was found to bind cDDX4 on the sensor chip in a dose-dependent manner (6.25, 12.5, and 25.0 nM) (A). For competition assay, IA2-F9 was pretreated with cDDX4 (1 and 10 nmol) (B) or linear DDX4 (1 and 10 nmol) (C) at room temperature prior to analysis. IA2-F9 competition was observed in the case of pretreatment with cDDX4 but not that of pretreatment with linear DDX4.

infected with HIV-1 X4 virus, LAV-1 (m.o.i. = 0.01) in the presence of IA2-F9 at varying doses, and the spread of infection was monitored based on the accumulation of p24 antigen in culture supernatants. IA2-F9 at 10 $\mu\text{g}/\text{ml}$ inhibited HIV-1 LAV-1 infection at the peak of virus production in the control experiment as shown in Fig. 7. These results indicate that cDDX4-MAP could induce the antibody with anti-HIV activity, and suggest the potential of immunization with cDDX4-MAP in AIDS therapy.

Immunogenicity of cDDX4-MAP in Cynomolgus Monkeys—To verify whether cDDX4-MAP could induce CXCR4-specific antibodies with anti-HIV-1 activity in nonhuman primates as well as rodents, an experiment was performed using cynomolgus monkeys immunized following the time schedule shown in Fig. 8A. Three cynomolgus monkeys were immunized with cDDX4-MAP in Freund's complete adjuvant or Freund's incomplete adjuvant by intraperitoneal or subcutaneous injection. Another three cynomolgus monkeys were immunized with MAP as the control. The cDDX4-immobilized BIAcore sensor chip was used for detecting cDDX4-specific antibodies in immunized monkeys. cDDX4-bound antibodies were detected in the sera from three monkeys 10 weeks after the initial immu-

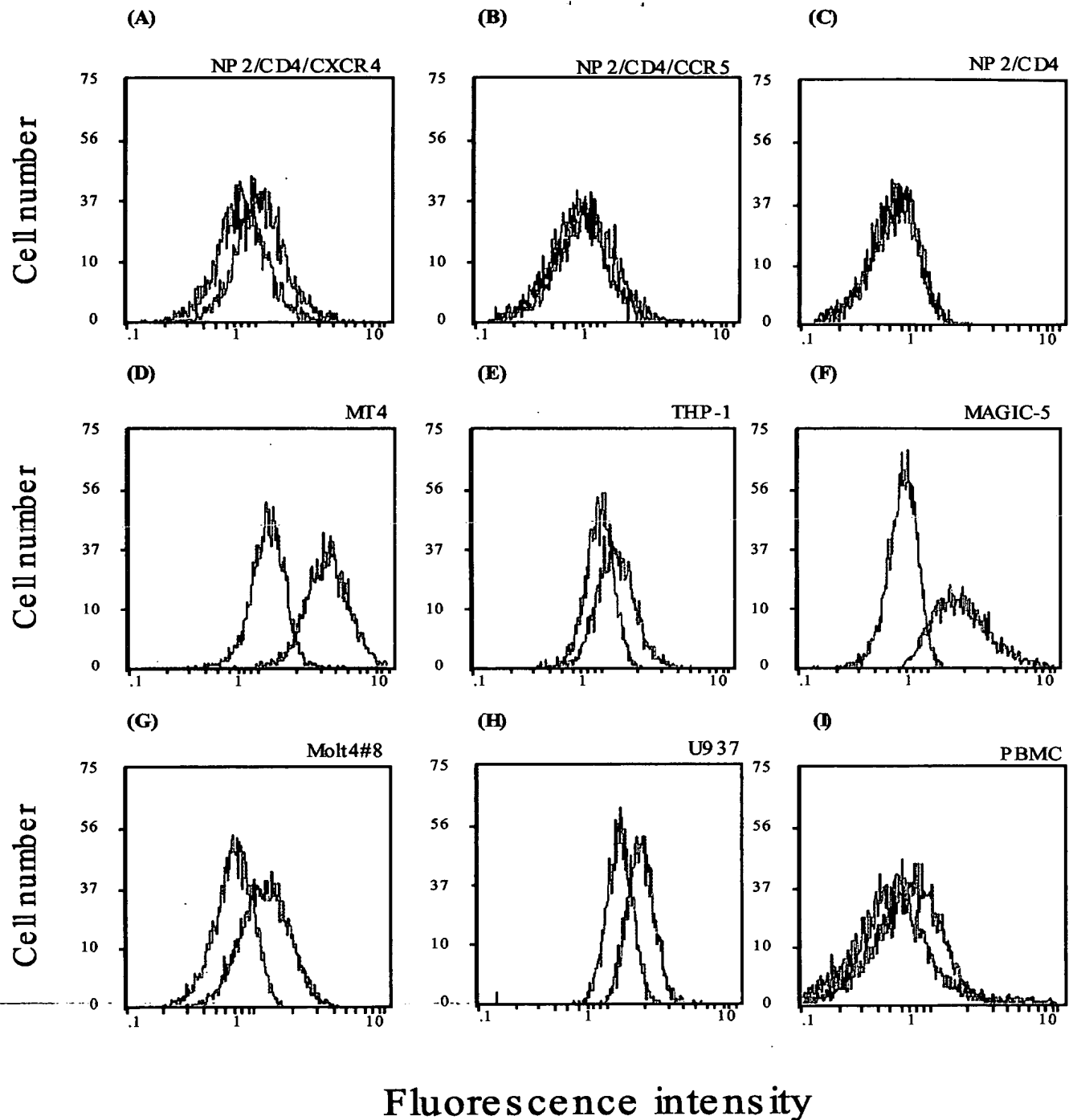


FIG. 4. Binding of IA2-F9 to cell-surface CXCR4. NP2/CD4/CXCR4 (A), NP2/CD4/CCR5 (B), NP2/CD4 (C), MT4 (D), THP-1 (E), MAGIC-5 (F), Molt4#8 (G), and U937 (H) cells, and PBMCs (I) were washed and suspended in cold washing buffer (PBS containing 2% fetal calf serum and 0.02% NaN_3). All samples were incubated with IA2-F9 (10 $\mu\text{g}/\text{ml}$) or isotype control for 30 min at 4 °C and subsequently incubated with FITC-conjugated anti-mouse IgM. IA2-F9 bound to all of the CXCR4-expressing cells.

nization (Fig. 8B). On the other hand, no significant responses were detected in MAP-immunized monkeys, which are the control (monkeys 4–6). These results indicate that cDDX4-MAP could induce CXCR4-specific antibodies not only in BALB/c mice but also in cynomolgus monkeys.

Anti-HIV Activity of Partially Purified Antibodies Derived from cDDX4-MAP-immunized Monkeys—Anti-HIV activity of antibodies that were induced by cDDX4-MAP was investigated. MAGIC-5 cells were inoculated with HIV-1 LAV-1 in the pres-

ence or absence of an Ig-containing fraction derived from the sera of cDDX4-MAP-immunized monkeys or MAP-immunized monkeys at 0 and 10 weeks. The Ig-containing antibodies from cDDX4-MAP-immunized monkeys (monkeys 1 and 2 in Fig. 9A) significantly inhibited HIV-1 infection but not those from monkey 3, as shown by the comparison between monkeys at 0 and 10 weeks. On the other hand, all sera from cDDX4-MAP immunized monkey at 27 weeks inhibited HIV-1 replication. The Ig-containing antibodies and sera from MAP-immunized mon-

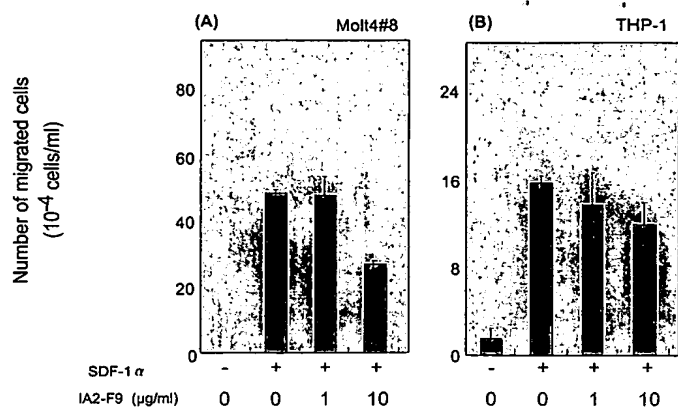


FIG. 5. **Chemotaxis assay.** Molt4#8 (A) and THP-1 (B) cells were pretreated with or without IA2-F9 for 30 min and were placed in the upper chamber of 5.0- μ m pore membranes (Corning). Chemotaxis was induced in the presence of 10 nM SDF-1 α (placed in the lower chamber). After incubation for 3 h at 37 °C, the cells migrating from the upper chamber to the lower chamber were quantified by trypan blue dye exclusion.

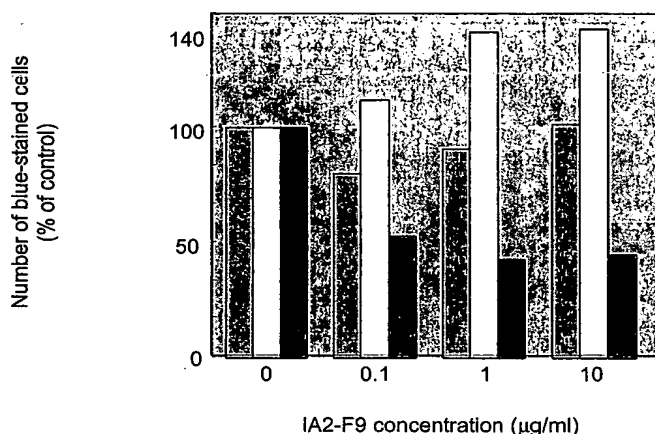


FIG. 6. **Effect of IA2-F9 determined by infection assay using MAGIC-5 cells.** MAGIC-5 cells (HeLa-CD4⁺CXCR4⁺CCR5⁺) were inoculated with the HIV-1 X4 virus LAV-1 (black), HIV-1 R5 virus JRFL (white), and HIV-1 R5X4 virus 89.6 (gray). Infection was conducted in the presence of DEAE-dextran and various concentrations of IA2-F9 (0–10 μ g/ml). After incubation for 48 h, the cells were fixed and stained with X-gal. The number of blue-stained cells is expressed as percentage (%) relative to the control. All data represent means \pm S.D. obtained from three separate experiments. No significant cytotoxicity of IA2-F9 was observed.

keys (monkeys 4–6 in Fig. 9, A and B) showed significantly enhanced infectivity of HIV-1 LAV-1 (Fig. 9A).

DISCUSSION

The chemokine receptor CXCR4 is a coreceptor for cellular entry of HIV-1 X4 virus and has been shown to be critical for development (17–19) but is probably dispensable in adults. However, when developing a self-antigen as target of an immunotherapeutic approach, unexpected effects must be taken into consideration. Therefore, for an immunotherapeutic approach to CXCR4, it is required to design an immunogen that targets a restricted region in CXCR4 rather than the whole CXCR4. A peptide immunogen strategy was used as an immunotherapeutic approach to CXCR4. Benefits of a peptide immunogen are generally as follows: (i) its immunogenicity can be controlled by polymerization or conjugation with small carrier molecules, (ii) it can induce antibodies against a very restricted region that includes the biologically active epitope, and (iii) its chemical purity can be exactly defined and is cost-effective to

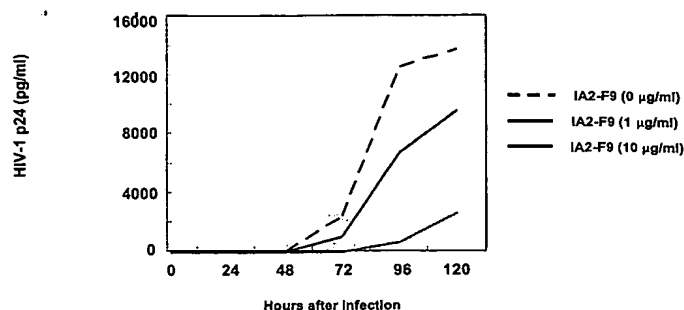


FIG. 7. **Effect of IA2-F9 determined by productive infection assay.** MT4 cells (1×10^5) were preincubated with various concentrations of IA2-F9 (0–10 μ g/ml) for 30 min, and then inoculated with HIV-1 X4 virus LAV-1 (m.o.i. = 0.01) for 2 h. After incubation for 72, 96, and 120 h, the p24 concentration in each supernatant was measured by antigen-capture enzyme-linked assay. All data represent means \pm S.D. obtained from three separate experiments. No significant cytotoxicity of IA2-F9 was observed.

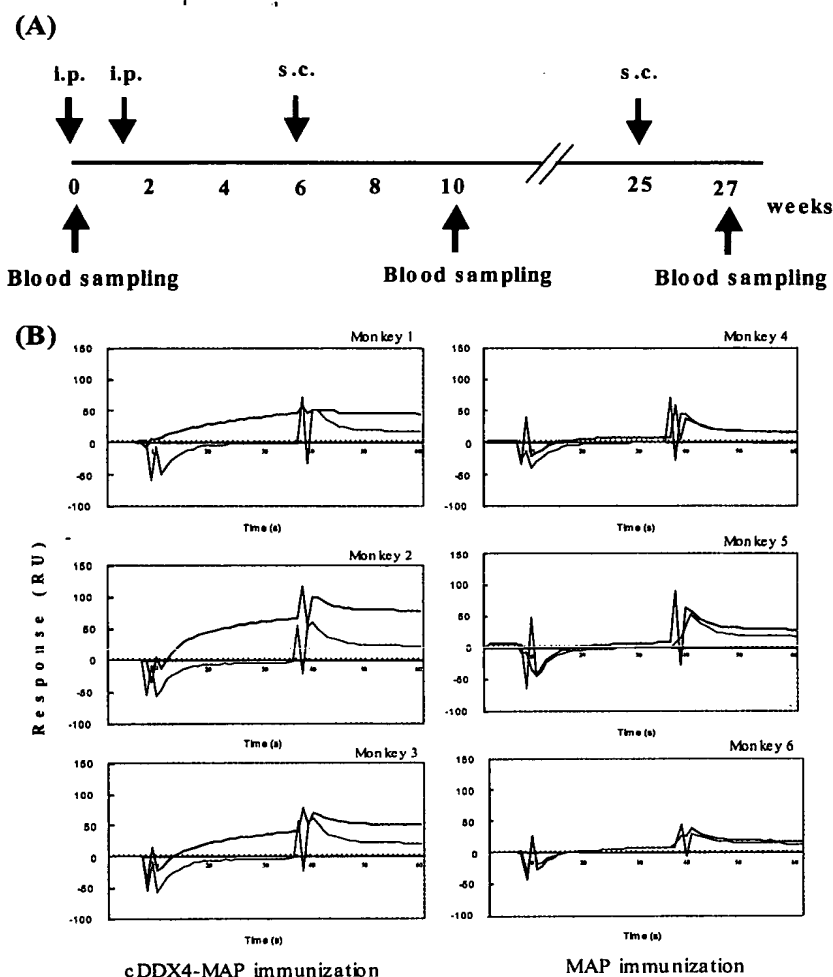
produce (20, 21). Therefore, the peptide immunogen strategy appears to be the best approach to inducing antibodies against CXCR4.

The peptide immunogen, cDDX4-MAP, was designed and prepared to mimic the conformational epitope of UPA in CXCR4, and antibodies were induced in BALB/c mice and cynomolgus monkeys. The UPA (from Asn¹⁷⁶ to Cys¹⁸⁶) of CXCR4 was selected as the target in the study because of its unique amino acid sequence. Amino acid sequence alignment shows that the amino terminus and the extracellular domains (ECL-1, ECL-2, and ECL-3) are homologous in the CXCR4 receptor family, but the amino acid sequence of UPA (from Asn¹⁷⁶ to Cys¹⁸⁶) of CXCR4 is specific (Table I, upper part, identical amino acid residues of UPA are shown in red), and highly conserved in the human, cynomolgus monkey, and rhesus monkey (Table I, bottom part). Furthermore, studies using site-directed mutagenesis of CXCR4 revealed that the amino acid residues Asp²⁰ and Tyr²¹ in the amino-terminal domain and Glu²⁶⁸ in ECL-3 are essential for SDF-1 α binding (31), whereas Glu¹⁷², Asp¹⁸¹, Asp¹⁸², Arg¹⁸³, and Tyr¹⁸⁴ in UPA had no effect on SDF-1 α binding as well as the intracellular Ca²⁺ influx (31, 33). Therefore, the immunogen designed based on UPA in CXCR4 may induce anti-CXCR antibodies with little effect on CXCR4 signaling. Consistent with our strategy, the monoclonal antibody induced by cDDX4-MAP inhibits weakly (Molt4#8 cell migration shown in Fig. 5) or moderately (THP-1 cell migration shown in Fig. 5) SDF-1 α -induced chemotaxis.

Mimicry of the conformation of the native protein is most important for inducing an antibody against the conformational epitope. Hence, an antibody induced by a linear peptide could recognize the denatured protein but could not recognize the native protein. On the other hand, an antibody that recognizes the conformational epitope of an antigen could not recognize the denatured protein. Indeed, the anti-CXCR4 antibody 12G5, induced by immunization of Sup-T1 cells that were chronically infected with SIVmac variant CP-MAC (23), could recognize cell surface CXCR4 as determined by fluorescence-activated cell sorting analysis but could not detect denatured CXCR4 as determined by Western blot analysis. To mimic the native conformational epitope of UPA in CXCR4, cDDX4 was prepared by cyclization of the decapeptide (¹⁷⁶NVSEADRYI¹⁸⁵) derived from the UPA sequence by insertion of the spacer-armed dipeptide (Gly-Asp). The spacer-armed dipeptide was used not only to conjugate cDDX4 to MAP but also to mimic the UPA in CXCR4 without inducing structural distortion.

The structural alignment between the hypothetical structural model of UPA in CXCR4, for which the structure of

FIG. 8. Immunization schedule and anti-cDDX4 antibody response in cynomolgus monkeys. *A*, immunization schedule for cynomolgus monkeys. Three cynomolgus monkeys (*Monkeys 1–3*) were immunized intraperitoneally (*i.p.*) at 0 and 1 week with 300 μ g of cDDX4-MAP, and boosted subcutaneously (*s.c.*) at 6 weeks with 300 μ g of cDDX4-MAP. Furthermore, these monkeys were reboosted at 25 weeks with 300 μ g of cDDX4-MAP. Another three cynomolgus monkeys (*Monkeys 4–6*) were immunized with MAP using the same schedule. Blood sampling was performed at 0, 10, 25, and 27 weeks. *B*, detection of anti-cDDX4 antibodies in serum derived from immunized cynomolgus monkey. The serum samples obtained at 0 week (*blue*) and 10 weeks (*black*) were dialyzed using Spectra/Por (molecular mass cutoff, 100 kDa; Spectrum Laboratories Inc.), and analyzed with BIAcore2000 using a cDDX4-immobilized sensor chip as described in Fig. 3.



rhodopsin was used as the template, and that of cDDX4 indicates that cDDX4 may possess the conformational property similar to that of an energy-minimized UPA structure. Because the conformational stability of a peptide antigen *in vivo* is also a key factor for generating antibodies against the native protein, the peptide antigen was cyclized not by a disulfide bond but by a peptide bond. Because the peptide bond is more robust and stable than a disulfide bond for cyclization, cDDX4 can maintain its conformation to a certain degree. Furthermore, as shown in the study of a thrombin-specific inhibitor, a cyclic peptide antigen seems to be more resistant to proteolytic degradation (34). Thus, cyclization of cDDX4 not only mimics the native conformational epitope of UPA in CXCR4, but also may enhance immunogenicity of cDDX4 itself by becoming resistant to proteolytic degradation.

The monoclonal antibody, IA2-F9, generated by immunization of BALB/c mice with cDDX4-MAP, showed conformational specific reactivity, as determined by BIAcore analyses, and specific binding to cell-surface CXCR4. These results indicate that IA2-F9 is a conformational epitope-specific antibody that cross-reacted with cell surface CXCR4. It is known that CXCR4 exhibits conformational heterogeneity (22), but IA2-F9 significantly reacted to all CXCR4-expressing cells that were used in this study. Although further investigation is required, UPA may be a conserved conformational region in CXCR4 that exhibits conformational heterogeneity. Furthermore, these results indirectly indicate that cDDX4 may accurately mimic the conformational epitope of UPA in CXCR4.

HIV-1 X4 virus emerges during chronic infection, and its emergence is associated with a rapid decline in the count of CD4(+) T cells and disease progression to AIDS (7–9). Recent statistical studies showed that long term nonprogressors have a high plasma SDF-1 α level and a low CXCR4 expression level on T lymphocytes, and in advancing disease, the expression level of CXCR4 increases and plasma SDF-1 α level decreases (16). In addition, some studies showed that the blockade of CXCR4 may prevent the emergence of X4 virus and change the phenotype of already-existing X4 virus to R5 virus (14, 15). Therefore, blocking CXCR4 is thought to be effective in inhibiting of progression to AIDS. IA2-F9 inhibited the replication of HIV-1 X4 virus as shown by the infection assay (MAGIC-5 assay, Fig. 6) and productive infection assay (HIV-1 p24 productive infection assay, Fig. 7). These results indicate that cDDX4 is the antigen that could induce anti-CXCR4 antibodies with anti-HIV-1 activity.

To investigate the immunogenicity of cDDX4-MAP in a non-human primate, cynomolgus monkeys were used in this study. Three cynomolgus monkeys were immunized four times with cDDX4-MAP. Immunization with cDDX4-MAP was well tolerated by all monkeys. No clinically significant changes in vital signs were observed in all monkeys immunized with cDDX4-MAP. In addition, there were no serious adverse events and toxicity for at least 1 year after immunization. Previous reports indicate the safety of the small-molecule CXCR4 inhibitors such as ALX40–4C and AMD3100 in humans (35, 36). Under limited conditions, these results support that the immunother-

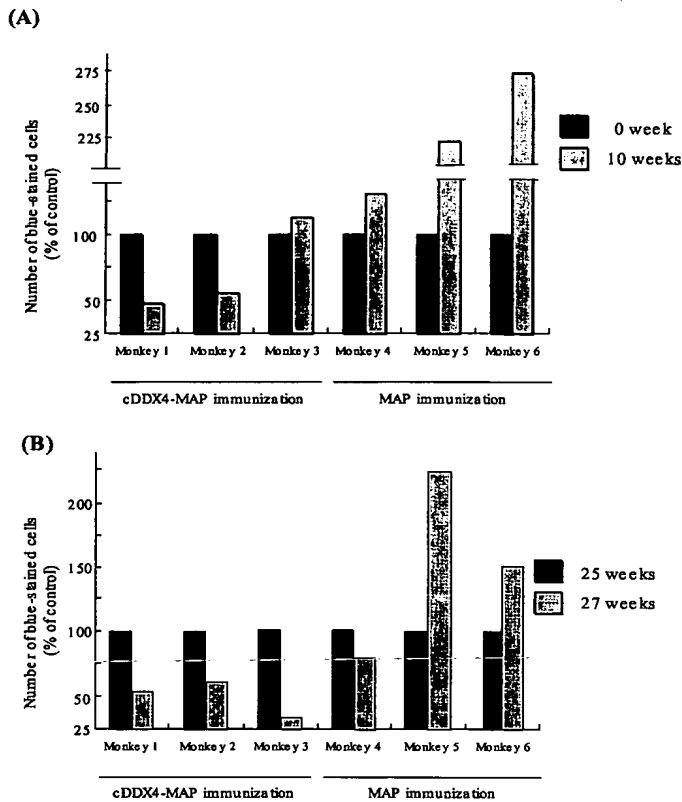


FIG. 9. Anti-HIV activity of serum antibodies derived from cDDX4-MAP immunized monkeys. MAGIC-5 cells (HeLa-CD4⁺CXCR4⁺CCR5⁺) were inoculated with the HIV-1 X4 virus LAV-1 in the presence of serum antibodies purified from the serum obtained at 0 week (black) or 10 weeks (gray) (A, upper figure), or the serum diluted at 1:10 serum obtained at 25 weeks (black) or 27 weeks (gray) (B, bottom figure). The number of blue-stained cells is expressed as percentage (%) relative to the number of blue-stained cells at 0 week for each monkey. No significant cytotoxicity of serum antibodies was observed.

apeutic approach using cDDX4-MAP is safe for application in nonhuman primates. However, it would be necessary to analyze the long term safety of this approach under various conditions in nonhuman primates in more detail, before this approach could be considered for human clinical trials. Because, unlike small-molecule compounds, additional effects, including induction of killer T cells, antibody-dependent cell-mediated cytotoxicity, and complement-dependent cytotoxicity (21, 37), should also be considered in the development of an immunotherapeutic approach.

Three monkeys produced antibodies to cDDX4-MAP as determined by BIAcore analyses, and at 10 weeks, the antibodies from monkeys 1 and 2 significantly inhibited HIV-1 infection as determined by MAGIC-5 assay, but not the antibodies from one monkey (monkey 3). However, at 27 weeks, the serum from monkey 3 also exhibited anti-HIV-1 activity. Although the reason for the difference in anti-HIV activity among the three monkeys is still unknown, the MAP moiety of the cDDX4-MAP antigen was shown to produce an unknown enhancer of HIV infectivity, because the enhancement of HIV infectivity was estimated by immunization with MAP alone. The balance in production of antibodies and an unknown enhancer, and individual differences among the monkeys may also account for differences in HIV-1 infectivity.

More recently, anti-HIV-1 agents to another chemokine receptor, CCR5, have been developed (24, 38), because individuals who lack CCR5 are resistant to HIV-1 infection and are healthy without any serious side effects (39–41). However,

TABLE I
Sequence alignment of undecapeptidyl arch in CXCR4 receptor family and among various species

Human	
CXCR1	A ₁₇₇ Y ₁₇₈ H ₁₇₉ P ₁₈₀ N ₁₈₁ N ₁₈₂ S ₁₈₃ S ₁₈₄ P ₁₈₅ V ₁₈₆ C ₁₈₇
CXCR2	T ₁₈₆ V ₁₈₇ Y ₁₈₈ S ₁₈₉ S ₁₉₀ N ₁₉₁ N ₁₉₂ S ₁₉₃ P ₁₉₄ A ₁₉₅ C ₁₉₆
CXCR3	H ₁₉₃ H ₁₉₄ D ₁₉₅ E ₁₉₆ R ₁₉₇ L ₁₉₈ N ₁₉₉ A ₂₀₀ T ₂₀₁ H ₂₀₂ C ₂₀₃
CXCR4	N ₁₇₆ V ₁₇₇ S ₁₇₈ E ₁₇₉ A ₁₈₀ D ₁₈₁ D ₁₈₂ R ₁₈₃ Y ₁₈₄ I ₁₈₅ C ₁₈₆
CXCR5	Q ₁₉₂ G ₁₉₃ H ₁₉₄ H ₁₉₅ N ₁₉₆ N ₁₉₇ S ₁₉₈ L ₁₉₉ P ₂₀₀ R ₂₀₁ C ₂₀₂
CXCR6	G ₁₇₀ N ₁₇₁ V ₁₇₂ F ₁₇₃ N ₁₇₄ L ₁₇₅ D ₁₇₆ K ₁₇₇ L ₁₇₈ I ₁₇₉ C ₁₈₀
Human	N V S E A D D R Y I C
Olive baboon	S V S E A D D R Y I C
Cynomolgus monkey	S V S E A D D R Y I C
Rhesus monkey	S V S E A D D R Y I C
Red-crowned mangabey	S V S E A D D R F I C
Sheep	D I K E A D E R Y I C
Cat	N V R E A D G R Y I C
Rat	D V S Q G D G R Y I C
Mouse	D V S Q G D D R Y I C
	I S Q G D

because individuals who progress to AIDS have X4 virus (1), development of a therapeutic strategy that targets CXCR4 is also indispensable (42–43). It is also known that individuals who have autoantibodies against CCR5 are resistant to HIV infection, although the induction mechanisms are still unclear (44). Therefore, we propose that an immunotherapeutic approach that uses cDDX4-MAP as a peptide immunogen may be a novel candidate strategy against HIV/AIDS progression.

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REFERENCES

- Berger, E. A., Murphy, P. M., and Farber, J. M. (1999) *Annu. Rev. Immunol.* **17**, 657–700
- Bieniasz, P. D., and Cullen, B. R. (1998) *Front. Biosci.* **3**, 44–58
- Moore, J. P., Trkola, A., and Dragic, T. (1997) *Curr. Opin. Immunol.* **9**, 551–562
- Littman, D. R. (1998) *Cell* **93**, 677–680
- Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) *Science* **272**, 872–877
- Berger, E. A., Doms, R. W., Fenyo, E. M., Korber, B. T., Littman, D. R., Moore, J. P., Sattentau, Q. J., Schuitemaker, H., Sodroski, J., and Weiss, A. (1998) *Nature* **391**, 240
- Scarlatti, G., Tresoldi, E., Bjorndal, A., Fredriksson, R., Colognesi, C., Deng, H. K., Malnati, M. S., Plebani, A., Siccardi, A. G., Littman, D. R., Fenyo, E. M., and Lusso, P. (1997) *Nat. Med.* **11**, 1259–1265
- Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S., and Landau, N. R. (1997) *J. Exp. Med.* **185**, 621–628
- Tersmette, M., de Goede, R. E., Al, B. J., Winkel, I. N., Gruters, R. A., Cuypers, H. T., Huisman, H. G., and Miedema, F. (1988) *J. Virol.* **62**, 2026–2032
- Gunthard, H. F., Frost, S. D., Leigh-Brown, A. J., Ignacio, C. C., Kee, K., Perelson, A. S., Spina, C. A., Havlir, D. V., Hezareh, M., Cooney, D. J., Richman, D. D., and Wong, J. K. (2000) *J. Virol.* **73**, 9404–9412
- Richman, D. D. (2001) *Nature* **410**, 995–1001
- Ramratnam, B., Mittler, J. E., Zhang, L., Boden, D., Hurley, A., Fang, F., Macken, C. A., Perelson, A. S., Markowitz, M., and Ho, D. D. (2000) *Nat. Med.* **1**, 82–85
- Takamune, N., Hamada, H., Misumi, S., and Shoji, S. (2002) *FEBS Lett.* **527**, 138
- Este, J. A., Cabrera, C., Blanco, J., Gutierrez, A., Bridger, G., Henson, G., Clotet, B., Schols, D., and De Clercq, E. (1999) *J. Virol.* **73**, 5577–5585
- Philpott, S., Weiser, B., Anastos, K., Kitchen, C. M., Robison, E., 3rd, Meyer, W. A., Sacks, H. S., Mathur-Wagh, U., Brunner, C., and Burger, H. (2001) *J. Clin. Invest.* **107**, 431–438
- Soriano, A., Martinez, C., Garcia, F., Plana, M., Palou, E., Lejeune, M., Arostegui, J. I., DeLazzari, E., Rodriguez, C., Barrasa, A., Lorenzo, J. I., Alcamí, J., DelRomero, J., Miro, J. M., and Gallart, T. (2002) *J. Infect. Dis.* **186**, 922–931
- Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T.,

- Bronson, R. T., Springer, T. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 9448–9453
18. Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T., and Nagasawa, T. (1998) *Nature* **393**, 591–594
19. Zou, Y. R., Kottmann, A. H., Kuroda, M., Taniuchi, I., and Littman, D. R. (1998) *Nature* **393**, 595–599
20. Arnon, R., and Horwitz, R. J. (1992) *Curr. Opin. Immunol.* **4**, 449–453
21. Dakappagari, N. K., Douglas, D. B., Triozzi, P. L., Stevens, V. C., and Kaumaya, P. T. (2000) *Cancer Res.* **60**, 3782–3789
22. Baribaud, F., Edwards, T. G., Sharron, M., Brelot, A., Heveker, N., Price, K., Mortari, F., Alizon, M., Tsang, M., and Doms, R. W. (2001) *J. Virol.* **75**, 8957–8967
23. Endres, M. J., Clapham, P. R., Marsh, M., Ahuja, M., Turner, J. D., McKnight, A., Thomas, J. F., Stoeckenau-Haggarty, B., Choe, S., Vance, P. J., Wells, T. N., Power, C. A., Sutterwala, S. S., Doms, R. W., Landau, N. R., and Hoxie, J. A. (1996) *Cell* **87**, 745–756
24. Misumi, S., Nakajima, R., Takamune, N., and Shoji, S. (2001) *J. Virol.* **75**, 11614–11620
25. Nardin, E. H., Oliveira, G. A., Calvo-Calle, J. M., Castro, Z. R., Nussenzweig, R. S., Schmeckpeper, B., Hall, B. F., Diggs, C., Bodison, S., and Edelman, R. (2000) *J. Infect. Dis.* **5**, 1486–1496
26. Galfre, G., and Milstein, C. (1981) *Methods Enzymol.* **73**, 3–46
27. Bernard, A., and Bosshard, H. R. (1995) *Eur. J. Biochem.* **230**, 416–423
28. MacKenzie, C. R., Hiram, T., Deng, S. J., Bundle, D. R., Narang, S. A., and Young, N. M. (1996) *J. Biol. Chem.* **271**, 1527–1533
29. Gosling, J., Monteclaro, F. S., Atchison, R. E., Arai, H., Tsou, C. L., Goldsmith, M. A., and Charo, I. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 5061–5066
30. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science* **289**, 739–745
31. Zhou, N., Luo, Z., Luo, J., Liu, D., Hall, J. W., Pomerantz, R. J., and Huang, Z. (2001) *J. Biol. Chem.* **276**, 42826–42833
32. Hachiya, A., Aizawa-Matsuoka, S., Tanaka, M., Takahashi, Y., Ida, S., Gatanaga, H., Hirabayashi, Y., Kojima, A., Tatsumi, M., and Oka, S. (2001) *Antimicrob. Agents Chemother.* **45**, 495–501
33. Brelot, A., Heveker, N., Montes, M., and Alizon, M. (2000) *J. Biol. Chem.* **275**, 23736–23744
34. Szewczuk, Z., Gibbs, B. F., Yue, S. Y., Purisima, E. O., and Konishi, Y. (1992) *Biochemistry* **31**, 9132–9140
35. Doranz, B. J., Filion, L. G., Diaz-Mitoma, F., Sitar, D. S., Sahai, J., Baribaud, F., Orsini, M. J., Benovic, J. L., Cameron, W., and Doms, R. W. (2001) *AIDS Res. Hum. Retrovir.* **17**, 475–486
36. Hendrix, C. W., Flexner, C., MacFarland, R. T., Giandomenico, C., Fuchs, E., J., Redpath, E., Bridger, G., and Henson, G. W. (2000) *Antimicrob. Agents Chemother.* **44**, 1667–1673
37. Ockert, D., Schmitz, M., Hampl, M., and Rieber, E. P. (1999) *Immunol. Today* **20**, 63–65
38. Lehner, T., Doyle, C., Wang, Y., Babaahmady, K., Whittall, T., Tao, L., Bergmeier, L., and Kelly, C. (2001) *J. Immunol.* **166**, 7446–7455
39. Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., Goedert, J. J., Buchbinder, S. P., Vittinghoff, E., Gomperts, E., Donfield, S., Vlahov, D., Kaslow, R., Saah, A., Rinaldo, C., Detels, R., and O'Brien, S. J. (1996) *Science* **273**, 1856–1862
40. Huang, Y., Paxton, W. A., Wolinsky, S. M., Neumann, A. U., Zhang, L., He, T., Kang, S., Ceradini, D., Jin, Z., Yazdanbakhsh, K., Kunstman, K., Erickson, D., Dragon, E., Landau, N. R., Phair, J., Ho, D. D., and Koup, R. A. (1996) *Nat. Med.* **2**, 1240–1243
41. Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A., and Landau, N. R. (1996) *Cell* **86**, 367–377
42. Rusconi, S., LaSeta-Catamancio, S., Citterio, P., Bulgheroni, E., Croce, F., Herrmann, S. H., Offord, R. E., Galli, M., and Hirsch, M. S. (2000) *J. Virol.* **74**, 9328–9332
43. Tremblay, C. L., Kollmann, C., Gigue, F., Chou, T. C., and Hirsch, M. S. (2000) *J. Acquir. Immune. Defic. Syndr.* **25**, 99–102
44. Lopalco, L., Barassi, C., Pastori, C., Longhi, R., Burastero, S. E., Tambussi, G., Mazzotta, F., Iazzarini, A., Clerici, M., and Siccardi, A. G. (2000) *J. Immunol.* **6**, 3426–3433

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1C-04

Cyclo-oligo peptideの化学合成とその生物活性

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庄司 省三

[目的]

環状ペプチドの化学合成とそれに対する特異抗体作出の試み。

[方法]

2種のケモカインレセプターのアミノ酸配列を含む12残基のドデカ保護ペプチドを合成し、そのペプチドのアミノ末端とカルボキシル末端を閉環縮合させ、環状ペプチドを調製した。この環状保護ペプチドの一部はそのまま脱保護し、ペプチドの性状決定に用い、他の一部は4分枝ポリリジン, multiple antigen peptide (MAP)に結合させ、免疫抗原として、残りをMulti-Pin法によりPinに結合させ、スクリーニング用抗原として用いた。ペプチドの環状の形成、ペプチド保護基の脱保護、ペプチドの純度はMatrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometer(MALDI-TOF MS, ドイツブルーカー社製)及びHPLCにより調べた。この環状ペプチドに対する単クローン抗体は、常法に従って調製した。抗体産生ハイブリドーマのスクリーニングは、上記のスクリーニング用抗原としてMulti-Pin法により合成した抗原ペプチドを用い、Multi-Pin-enzyme linked immunosorbent assay法(Multi-Pin-ELISA法)により抗体のスクリーニングを行なった。

[結果・考察]

ドデカ保護ペプチド (Asp-Ser-Gln-Lys-Glu-Gly-Glu-Ala-Asp-Asp-Arg-Gly)を化学合成し、そのアミノ末端とカルボキシル末端はBenzotriazolyloxytris(dimethylamino) phosphonium Hexafluorophosphate(BOP)で効率良く閉環することができ、環状ペプチド合成に成功した。その環状ペプチドの収率は50-60%であった。この環状形成はMALDI-TOF MSスペクトラム上、水分子脱離により質量数18の減少により確認することができた。この環状ペプチドをMAPに結合して、単クローン抗体作製用の免疫抗原とした。さらに環状ペプチドを結合したMulti-Pin-ELISA法により数種の抗体産生細胞を得て、現在単クローン化を行っており、今後、得られた単クローン抗体の免疫学的諸性質を調べる予定である。

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1C-04

Chemical synthesis of a cyclo-oligo peptide and its biological activity

Kumamoto University Faculty of Pharmaceutical Sciences

○Yasuhide IDO, Shogo MISUMI, Kazuchika FURUISHI, and

Shozo SHOJI

[Objective]

Chemical synthesis of a cyclic peptide and attempt to produce its specific antibodies.

[Method]

A protected dodecapeptide composed of 12 residues and containing amino acid sequences of two chemokine receptor was synthesized, and its amino and carboxyl termini were subjected to condensation for ring closure. A portion of the thus-prepared protected cyclic peptide was deprotected and used for characterization of the peptide. Another portion was bonded to four-branched polylysine, a multiple antigen peptide (MAP), for use as an immunogen, and the remaining portion was bonded to pins by the Multi-Pin Method for use as an antigen for screening. The peptide ring formation, peptide protecting group elimination, and peptide purity were examined by using a Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometer (MALDI-TOF MS, product of Bruker, Germany) and by HPLC. Monoclonal antibodies to this cyclic peptide were prepared in the conventional technique. Screening for antibody-producing hybridomas was carried out by the Multi-Pin-enzyme linked immunosorbent assay (Multi-Pin-ELISA) using the above antigen peptide, synthesized by the Multi-Pin Method, as the antigen for screening.

[Results and Discussion]

A protected dodecapeptide (Asp-Ser-Gln-Lys-Glu-Gly-Glu-Ala-Asp-Asp-Arg-Gly) was chemically synthesized, and the linkage of the N- and C-terminus of the dodecapeptidyl linear chain could be efficiently achieved by using benzotriazolyloxytris (dimethylamino) phosphonium hexafluorophosphate (BOP). As a result, the corresponding cyclic peptide was thus successfully obtained in a 50-60% yield. The ring formation could be confirmed by decrease of 18 in mass number resulting from elimination of H₂O on a MALDI-TOF MS spectrum. This cyclic peptide was bonded to the MAP for use as an immunogen for monoclonal antibody production. Furthermore, several antibody-producing hybridomas were obtained by screening with the Multi-Pin-ELISA. The hybridomas are now being cloned repeatedly and thereafter the obtained clones are checked with respect to their immunological properties.

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SPECIFICATION

CYCLIC PEPTIDES AND AIDS VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a National Stage Entry of International Application No. PCT/JP97/06174, filed November 5, 1997, and claims priority of Japanese Application No. 11-32990, filed February 10, 1999.

5 FIELD OF THE INVENTION

The present invention relates to cyclic peptides effective in preventing HIV-1 virus infection in human and to AIDS vaccines. More particularly, it relates to cyclic peptides which serve as antigens for producing a neutralizing antibody capable of neutralizing HIV-1 virus infection via the second receptors called CXCR4 and CCR5 and to AIDS vaccines which

10 comprise the above antigens as active ingredients.

BACKGROUND OF THE INVENTION

15 Second receptors which the pathogenic virus causative of AIDS (HIV-1 virus) utilizes in infecting human were identified in 1996 (Yu Feng et al., Science, 272, 872-877, 1996). These receptors are two receptors ^{are} called CXCR4 and CCR5 among the chemokine receptors already reported. It has been revealed that

20 the HIV-1 virus utilizes one of the receptors for adsorption onto and entry into lymphocytes, macrophages and dendritic cells to achieve infection.

On the other hand, about 1 to 2% of Caucasians reportedly have resistance to HIV-1 virus infection and it has been

25 revealed that this is due to a genetic defect or genetic incompleteness of the second receptors (CXCR4 and CCR5), which are chemokine receptors (Rong Liu et al., 86, 367-377, 1996).

These findings have called researchers' attention to the importance of neutralization of the second receptors in the prevention of HIV-1 virus infection and, in recent years, attempts have been made to produce a neutralizing antibody capable of neutralizing the second receptors. There is no report, however, ^{as} about the successful creation of such a neutralizing antibody.

Accordingly, it is an object of the present invention to provide three-dimensional antigens capable of producing, in vivo, a neutralizing antibody capable of neutralizing the second receptors from the stereoscopic viewpoint by paying attention to the loop structures of the second receptor proteins without following the conventional methods ^{which} of interpreting the peptides constituting the second receptors two-dimensionally.

Another object is to provide AIDS vaccines which comprise such antigens as active ingredients.

DISCLOSURE OF THE INVENTION

The present inventors constructed a model of the second receptor in T cells (abbr.: CXCR4) and a model of the second receptor in macrophages (abbr.: CCR5) and observed them from ^a the three-dimensional viewpoint. As a result, they explored the applicability of two pentapeptides constituting the second subloop (UPL) in the respective second receptor proteins, namely

T cell-derived Glu₁₇₉-Ala₁₈₀-Asp₁₈₁-Asp₁₈₂-Arg₁₈₃ ^(Seq. ID No 2) and
macrophage-derived Ser₁₈₈-Gln₁₇₀-Lys₁₇₁-Glu₁₇₂-Gly₁₇₃ ^(Seq. ID No 3) as
constituent elements of a novel antigen for producing an HIV-1

virus infection-preventing antibody capable of neutralizing the second receptors. ~~And, as a result, they have now completed the present invention.~~

Thus, the present invention ^{provides} ~~consists in~~ a cyclic peptide which is a novel compound and ^{which} comprises, as a constituent chain thereof, one or two amino acid sequences selected from among amino acid sequences contained in the second subloop in the T cell second receptor protein and comprising at least five amino acid residues and amino acid sequences contained in the second subloop in the macrophage second receptor protein and comprising at least five amino acid residues. ^{The present invention further provides} ~~as well as in~~ AIDS vaccines comprising that compound as ^{an} active ingredients.

More specifically, the cyclic peptide of the present invention, which is a novel compound, is characterized in that it comprises one or two amino acid sequences selected from the groups consisting of the amino acid sequence Glu-Ala-Asp-Asp-Arg₁ ^(Seq ID No 2) and the amino acid sequence Ser-Gln-Lys-Glu-Gly₁ ^(Seq ID No 3) as a constituent chain or chains thereof, and the AIDS vaccine is characterized by comprising such compounds as active ingredients.

More particularly, the cyclic peptide of the invention is characterized in that it is a novel compound which is represented by the formula (1) given below and the AIDS vaccine of the invention is characterized in that it comprises that compound as an active ingredient.

Arg-Asp-Asp-Ala-Glu-Gly

|

|

Formula (1)

Gly-Asp-Ser-Gln-Lys-Glu

(Seq ID No. 1)

5 Fig. 1 shows the configuration of a T cell-derived second
receptor protein molecule on the T cell membrane (Fig. 1, top
left) and the configuration of a macrophage-derived second
receptor protein molecule on the macrophage membrane (Fig. 1,
top right) and a cyclic dodecapeptide according to the
10 invention as synthesized from the respective second subloop
peptides of these second receptor protein molecules. In Fig. 1,
the T cell-derived second receptor protein molecule (CXCR4) has
a configuration comprising a first loop, a second loop, a third
loop and a second subloop and the macrophage-derived second
15 receptor protein molecule (CCR5) also has a configuration
comprising a first loop, a second loop, a third loop and a
second subloop.

The second subloop in the T cell-derived second receptor
protein molecule (CXCR4) contains the amino acid sequence Glu₁₇
20 -Ala₁₈₀-Asp₁₈₁-Asp₁₈₂-Arg₁₈₃ (Seq ID No. 2) and the second subloop in the
macrophage-derived second receptor protein molecule (CCR5)
contains the amino acid sequence Ser₁₆₉-Gln₁₇₀-Lys₁₇₁-Glu₁₇₂-
Gly₁₇₃. (Seq ID No. 3)

A novel compound cyclic dodecapeptide of the present
25 invention as represented by the formula (1) shown above (cyclic
peptide shown in Fig. 1, bottom) can be obtained by causing
both the peptides respectively having the above-identified

amino acid sequences of both the second subloops of CXCR4 and CCR5 to form a ring via -Gly-Asp- as a spacer arm dipeptide.

Preferably, an active group selected from among the carboxyl, amino and hydroxyl groups contained in the cyclic dodecapeptide represented by the above formula (1) is bonded to a substituent group so that the absorption into the living body and antibody formation may be facilitated. Such a substituent can be selected from among the residues of [a] fatty acid $\text{CH}_3(\text{CH}_2)_n-\text{COOH}$ (n: 0 to 20), the residues of [an] alcohol $\text{CH}_3(\text{CH}_2)_n-\text{OH}$ (n: 0 to 20) and the unsaturated compound residues corresponding to such compound residues and preferably has biocompatibility. As appropriate examples of the fatty acid, there may be mentioned [a] lauric acid, [a] myristic acid, [a] palmitic acid, [a] stearic acid, [an] arachidonic acid, and unsaturated fatty acids corresponding thereto. As appropriate higher alcohols, there may be mentioned [a] lauryl alcohol, [a] myristyl alcohol, [a] palmityl alcohol, [a] stearyl alcohol, [an] eicosanol, and unsaturated alcohols corresponding thereto.

The cyclic dodecapeptide represented by the above formula (1) can be utilized as an immunogen for producing a second receptor neutralizing antibody capable of inhibiting HIV-1 virus infection. ~~[In the following, mentioned is made of that]~~ immunogen.]

An assaying antigen for antibody screening is prepared by binding the cyclic dodecapeptide to a solid phase resin. Separately, mice were immunized with an immunogen, for example a cyclic dodecapeptide-multiple antigen peptide (abbr.: CDP-MAP),

and monoclonal antibodies are prepared by the conventional hybridoma technique. For confirming the anti-infective activity against HIV-1 virus infection, several hybridomas (^{fusion of} fused) cells ^{produced by} between antibody-producing B cells and myeloma cells (cancer cells)) are prepared by the above method and anti-HIV-1 virus activity assaying is carried out in the conventional manner using the hybridoma culture supernatants, ^{to show} whereby the culture supernatants prevent HIV-1 virus infection. ✓

Thus, the cyclic dodecapeptide represented by the formula (1) can be used as an immunogen for producing antibodies having inhibitory effects against HIV-1 virus infection and therefore is useful as ^{an} active ^{agent} ingredients in AIDS vaccines. ✓

The AIDS vaccines according to the invention ^{contain} can comprise, as ^{an} active ^{agent} ingredients, a cyclic peptide comprising, as a constituent chain or chains thereof, one or two amino acid sequences selected from the amino acid sequence Glu-Ala-Asp-Asp-Arg, ^(Seq ID No 2) and the amino acid sequence Ser-Gln-Lys-Glu-Gly, ^(Seq ID No 3)

The AIDS vaccines according to the invention may comprise the above cyclic peptides as active ^{agents} ingredients or the active ^{agents} ingredients may be ^{made} [a] modification ^{of} derived from the cyclic peptides by substitution and/or addition or may be in the form of a pharmacologically acceptable salt. [The] pharmacologically acceptable salt ^s includes salts with hydrochloric acid, [a] sulfuric acid, [a] nitric acid, [a] nitrous acid, [a] hydrobromic acid, [a] hydroiodic acid, [a] phosphoric acid and organic acids.

An example of ^{a suitable derivative} the (modification) of the compound of the above formula (1) in which the substituent group is a higher fatty

^{is that}

acid group²⁵ is shown below.

Arg - Asp - Asp - Ala - Glu - Gly

5 Gly - Asp - Ser - Gln - Lys - Glu

Formula (2)

(Seq. ID No. 1)

MAP

Five equivalents of 9-fluorenylmethoxycarbonyl-
10 dimethylsulfonium methyl sulfate (Fmoc-DSP; tradename, product
of Novabiochem) are added to 1 equivalent of the cyclic
dodecapeptide-MAP represented by the formula (2) to thereby
block the ϵ -amino group of K₄ of the cyclic dodecapeptide-MAP
and then the carboxyl groups (E₅, E₇, D₉, D₁₀) are activated
15 with EDC, DCC, BOP or the like, and a higher alcohol [CH₃(CH₂)_n
-OH] is added in excess to thereby effect esterification. *Alternatively,*
the hydroxyl group of Ser of the cyclic dodecapeptide-MAP
represented by the above formula (2) is esterified by the acid
chloride [CH₃(CH₂)_nCOCl] method and, after elimination of Fmoc,
20 the ester is used as a base material of the peptide vaccine.
When the vaccine is administered to ^{or}the living body, it is
delivered to lymphoid tissues, where the ester is hydrolyzed.
The thus-recovered original cyclic peptide-MAP represented by
the formula (2) activates the immune system, whereby antibodies
25 are produced and the AIDS virus infection is neutralized.

The AIDS vaccines according to the invention can be used as
a pharmaceutical compositions in the form or oral or nonoral

preparations. The oral dosage form includes tablets, powders, granules, capsules, microcapsules, solutions and the like. The nonoral or parenteral dosage form includes solutions, mainly injectable solutions, and suppositories, among others.

5 Generally, these preparations may contain one or more of pharmaceutical preparation auxiliaries such as carriers, excipients, binders, disintegrants, lubricants, stabilizers, flavors, and the like.

The dose thereof may vary according to the symptom and/or
10 age. In the case of oral administration, a daily dose of 0.1 to 1000 mg/kg body weight can be administered to ^{normal} ordinary adults.

BEST MODES FOR CARRYING OUT THE INVENTION

Example 1

15 (1) Synthesis of a cyclic chimera peptide comprising second subloop peptides of two types of receptors for HIV-1

The resin used for solid synthesis of the peptide was a 2-chlorotrisyl chloride resin, which will not impair the protective groups on various amino acid residues and from which
20 the peptide can be cleaved with a weak acid. A 0.25-mmol (368-mg) portion of the resin was weighed and used. The peptide synthesis was carried out according to the Fmoc (9-fluorenylmethoxycarbonyl) chemistry and a Fmoc-side chain-protected peptide-resin was obtained by starting the synthesis
25 from the C terminus on a fully automated peptide synthesizer using the following Fmoc-side chain-protected amino acids 1) to 12) (1.0 mmol each).

- 1) Fmoc-Gly-OH 1.0 mmol
- 2) Fmoc-L-Arg(Pmc)-OH 1.0 mmol
Pmc: 2, 2, 5, 7, 8-pentamethylchroman-6-sulfonyl
- 3) Fmoc-L-Asp(OtBu)-OH 1.0 mmol
- 5 OtBu: O-t-butyl
- 4) Fmoc-L-Asp(OtBu)-OH 1.0 mmol
- 5) Fmoc-L-Ala-OH 1.0 mmol
- 6) Fmoc-L-Glu(OtBu)-OH 1.0 mmol
- 7) Fmoc-Gly-OH 1.0 mmol
- 10 8) Fmoc-L-Glu(OtBu)-OH 1.0 mmol
- 9) Fmoc-L-Lys(Boc)-OH 1.0 mmol
Boc: benzyloxycarbonyl
- 10) Fmoc-L-Gln(Trt)-OH 1.0 mmol
Trt: trityl
- 15 11) Fmoc-L-Ser(tBu)-OH 1.0 mmol
tBu: t-butyl
- 12) Fmoc-L-Asp(OBzl)-OH 1.0 mmol
OBzl: O-benzyl

20 The protected peptide resin (300 mg) obtained in the above process was admixed with 5 ml of an acetic acid/trifluoroethanol/dichloromethane (1:1:8) mixture, the mixture was stirred at room temperature for 30 minutes and then filtered to thereby separate the side chain-protected peptide liberated with the weak acid from the resin, and ether was added to the filtrate in

25 the conventional manner. To the thus-obtained precipitate was added an appropriate amount of acetonitrile, followed by lyophilization. By causing the carboxyl group of the C

terminal Gly of this side chain-protected dodecapeptide to condense with the amino group of the amino terminal Asp(OBzl) thereof, a cyclic dodecapeptide was synthesized as follows.

5 The side chain-protected linear dodecapeptide (130 mg) was dissolved in 80 ml of a dimethylformamide solution containing 10% trifluoroethanol, 5 times the amount of the peptide of benzotriazol-1-yloxytris(dimethylamino)phosphonium
hexafluorophosphate (abbr.: BOP), the mixture was allowed to stand at room temperature for 24 hours to thereby allow the
10 reaction to proceed, and 80 mg of a side chain-protected cyclic peptide was recovered by the conventional method.

This side chain-protected cyclic dodecapeptide was dissolved in 10 ml of dimethylformamide, 50 mg of palladium-carbon was added, catalytic reduction was carried out using hydrogen gas
15 for 24 hours, and a carboxymethyl side chain-protected cyclic dodecapeptide (15 mg) was obtained by the conventional method.

For identifying the cyclic dodecapeptide, all the protective groups were eliminated in the conventional manner and laser mass spectrometry was performed (MALDI-TOF mass spectrometer).
20 The theoretical values and measured values for the cyclic peptide and linear (noncyclic) peptide are given below in Table 1. In Fig. 2, the MALDI TOF mass spectra for the cyclic peptide and linear (noncyclic) peptide are shown. The cyclic dodecapeptide was thus identified based on the results shown
25 (reduction by molecular mass of water 18 as a result of dehydration condensation under ring formation).

Table 1

	Mass	Theoretical value	Measurement value
5 Cyclic peptide	1287.53	1288.53	1288.54
Linear (noncyclic) peptide	1305.54	1306.55	1306.73

(2) Preparation of immunogen comprising cyclic dodecapeptide-

10 MAP (abbr.: CDP-MAP)

The carboxyl group of the carboxymethyl side chain-blocked cyclic dodecapeptide (abbr.: CM-SBCDP) was condensed with the amino group of tetra-branching polylysine of a MAP resin by the BOP method, as follows.

15 70 mg (32 μ mol) of the MAP-resin (0.46 mmol tetra-branching polylysine/resin) was swelled in dimethylformamide (DMF) and the MAP-resin was deprotected (elimination of Fmoc) three times with 10 ml of 20% piperidine/dimethylformamide, washed three times with 5-ml portions of isopropanol and then ^{separated from} deprived of

20 the isopropanol, to expose the amino terminus of the tetra-branching polylysine. To this MAP-resin was added 10 ml (32 μ mol) of a solution of the carboxymethyl side chain-blocked cyclic dodecapeptide in dimethylformamide and the binding between them was effected by the BOP method. The peptide was

25 cleaved from the side chain-blocked cyclic dodecapeptide (abbr.: SBCDP)-MAP-resin in the conventional manner by treatment with trifluoroacetic acid (abbr.: TFA), whereby 12 mg of the

cyclic dodecapeptide-MAP (abbr.: CDP-MAP) was obtained. This was used as an immunogen for preparing anti-cyclic dodecapeptide (abbr.: Anti-CDP) monoclonal antibodies.

- (3) Preparation of CDP-pin resin (crown resin) as assaying
5 antigen for preparing anti-cyclic dodecapeptide (Anti-CDP) monoclonal antibodies

The assaying antigen for efficiently producing anti-CDP monoclonal antibodies from culture supernatants was prepared in the following manner. The side chain-blocked cyclic
10 dodecapeptide was bound to β -Ala at the pointed end of the pin resin (crown resin) according to the epitope scanning kit manual (Chiron Mimotopes Pty Ltd, Clayton, Victoria, Australia) to give a CDP-pin resin (crown resin).

- (4) Preparation of monoclonal antibody-producing hybridomas

- 15 Balb/c mice were primarily immunized using the cyclic dodecapeptide-MAP as the immunogen peptide and cell fusion was carried out in the conventional manner using myeloma cells (P3U1) and polyethylene glycol. After fusion, selective culture was carried out using HAT medium and, for the wells in which
20 hybridoma cells formed colonies, the antibody titer in each culture supernatant was determined by the multi-pin ELISA method using the antigen peptide. For each cell group judged as antibody-positive, cloning was performed twice by limiting dilution and a monoclonal antibody-producing hybridoma line was
25 established by the conventional method. For basal immunization, the lyophilized immunogen peptide was dissolved in PBS(-) to a concentration of 1 mg/ml and this solution was admixed, at a

ratio of 1:1.2 to 1:1.4, with the immunostimulator Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA), and the thus-prepared emulsion was used. This emulsion was intraperitoneally administered at a dose of 400 μ l/mouse four
5 times in total at one-week intervals. For the first two administrations, an emulsion with FCA was used and, for the last two administrations, an emulsion with FIA was used. The final or boost immunization was carried out after the lapse of one month following completion of the basal immunization by
10 intravenous administration, through the caudal vein, of a 200 μ g/ml solution of the lyophilized immunogen peptide (MAP) in PBS(-) at a dose of 200 μ l/mouse.

① Preparation of splenic cells and cell fusion

The preparation of splenic cells and cell fusion were carried
15 out in the conventional manner. Three or four days after the final immunization, mice were sacrificed by exsanguination, splenocytes were excised and loosened in Hank's balanced salt solution (HBSS) and deprived of erythrocytes by hemolytic buffer treatment and centrifugation. The splenic cells thus prepared
20 were mixed with P3U1 cells at a ratio of P3U1: splenic cells = 1:8 to 1:10 and the mixture was centrifuged. A polyethylene glycol solution was added to the pellet obtained to thereby effect fusion. After fusion treatment, the fused cells were
25 gently suspended in HAT medium and the suspension was distributed in the wells of 48-well plates and cultured at 37°C until the fused cells formed colonies.

② Screening for antibody-producing hybridomas

Screening for specific antibody-producing hybridomas was effected and the desired hybridomas were selected by continuously carrying out primary screening by the ELISA method using the immunogen peptide as a solid phase antigen and secondary screening using the multi-pin peptide as a solid phase antigen. In ELISA, the hybridoma culture supernatant was used as a primary antibody, peroxidase (POD)-labeled anti-mouse IgG as a secondary antibody, TMBZ (3,3',5,5'-tetramethylbenzidine) as a color substrate, and 0.3 N H₂SO₄ as color development stop solution, and the absorbances were measured at a dominant wavelength of 450 nm and at a reference wavelength of 630 nm.

③ Cloning of a desired antibody-producing hybridoma line

A monoclonal hybridoma strain showing high antibody titer in the screening assay was subjected to limiting dilution to one cell/well. The thus-cloned cells were distributed, together with feeder cells prepared from the murine thymus, into the wells of 96 well plates and cultured. After two repetitions of this cloning procedure, the group of monoclonal cells was subjected to screening by multi-pin ELISA using the antigen peptide. The cell line which showed the highest antibody titer in both ELISA screenings was selected as the monoclonal antibody-producing hybridoma line and the monoclonal antibody was purified from the culture supernatant thereof in the conventional manner. The subclass of this monoclonal antibody was found to be IgM κ . This hybridoma was deposited on February 3, 1998 with the Agency of Industrial Science and Technology National Institute of Life Science and Human

Technology under the accession number FERM P-17198 and this
deposition²¹ was transferred on October 27, 1998 to the
international deposition²¹ under the Budapest Treaty under the
accession number FERM BP-6925. The cell line established was
5 extended and cultured and the cells were frozen^{and} stored in a
liquid nitrogen tank.

(5) Anti-HIV activity assay

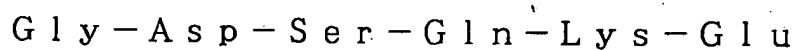
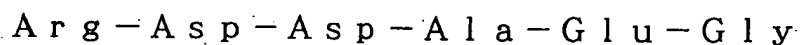
The anti-HIV activity was measured by the method of Maeda et
al. (Y. Maeda, et al., 12th World AIDS Conference Geneva,
10 Abstract P4, June 28-July 3, 1998). The culture fluid of the
anti-CDP monoclonal antibody-producing cells created by the
present inventors and that of the corresponding non-antibody-
producing cells as a control as obtained under the same
conditions were used. The antibody-containing culture fluid
15 (200 μ l) reduced the rate of infection with HIV-1 virus to 61%
in 30 minutes and to 35% in 60 minutes as compared with the
control and thus was established that it inhibits the
infectivity of HIV-1 virus.

20 INDUSTRIAL APPLICABILITY

The cyclic peptide of the invention is a novel compound and
is useful as an antigen for producing, in vivo, a neutralizing
antibody (antibody having an anti-HIV-1 virus activity) capable
of neutralizing the HIV-1 virus infection via the second
25 receptor called CXCR4 and/or CCR5. It is also useful as an
active ^{agent} ingredient of an AIDS vaccine.

ABSTRACT

Cyclic peptides comprising, as a constituent chain or chains, one or two amino acid sequences selected from the groups consisting of the amino acid sequence Glu-Ala-Asp-Asp-Arg and the amino acid sequence Ser-Gln-Lys-Glu-Gly, and AIDS vaccines containing the cyclic peptide as an active ingredient. Preferably a cyclic dodecapeptide represented by the formula given below and an AIDS vaccine containing the cyclic dodecapeptide as an active ingredient. From the in vivo absorption and antibody formation viewpoint, active groups selected from among the carboxyl, amino and hydroxyl groups contained in the cyclic peptide is preferably bound to substituent groups. The cyclic dodecapeptide can neutralize the second receptors in the infection of human with HIV-1 virus.



(Seq. ID No. 1)